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Compound-Specific Stable Isotopic Analysis of Protein Amino Acids: Ecological Applications in Modern and Ancient Systems

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by

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Table of Contents

List of Tables

List of Figures

Abstract

Stable isotopic analysis of the major biochemically important elements is an increasingly utilized tool in the study of ecology. Patterns of isotopic fractionation in carbon and nitrogen are used to determine trophic linkages, nutrient pathways, and sources of primary production in numerous contexts. Traditional techniques rely on measurements made of bulk samples such as tissue, but emerging methods using individual chemical compounds provide a means of achieving deeper understanding in a variety of inquiries.

Amino acids, as the building blocks of proteins, are the dominant nitrogen-bearing biomolecules and are a major constituent of all life. Patterns of isotopic fractionation during synthesis and transformations of these compounds record a variety of information about their environmental history. The purpose of this study was to utilize amino acid-specific isotopic analysis to address a variety of questions in paleoecology and trophic ecology, with an eye towards overcoming limitations inherent in bulk analyses applied to these fields.

Organic matter preserved in shells provides an archive of compounds that are typically lost quickly from the environment, such as proteins. It can be used as an analog of the soft body parts typically used isotope-based environmental measures and therefore provide a window on past environmental conditions, if it can be demonstrated

that the two are chemically equivalent. Compositional differences between tissue and shell organic matter can obscure this relationship in bulk analyses, so a compound-specific approach is needed to accurately test this idea. Comparison of amino acid $\delta^{13}C$ values between hinge muscle tissue and shell organic matter in *Crassostrea virginica* sampled along an estuarine salinity gradient in Rookery Bay, Florida, demonstrated functional equivalency between them. While minor isotopic offsets were observed between them, likely due to differences in turnover time between tissues, the ability to resolve location within the estuary was identical between them. This suggests that amino acid-specific isotopic measurements from shell organic matter can be effectively used in paleoreconstructions.

A complicating factor in using shell organic matter as a surrogate for body tissues arises from interspecies differences in organic matrix composition. This manifests itself as species-specific isotopic offsets in bulk analyses, making meaningful comparisons across species difficult. Amino acid nitrogen isotope analysis of a suite of mollusks from St Joe Bay, Florida, was used to infer trophic positions from shell organics and the results obtained were compared to equivalent tests using body tissue. Despite the inability to reconstruct trophic position from bulk isotopic compositions, shell organic extracts produced an identical description of trophic levels to that obtained using body tissues. CSIA, therefore, can be used to eliminate compositional biases in interspecies comparisons using organic matter preserved in biominerals.

Trophic level determinations derived from measurements of amino acid $\delta^{15}N$ benefit from that fact that a subset of these compounds directly record ecosystem isotopic baseline values. It is therefore possible to parse the source of observed changes in bulk isotopic compositions into contributions from both baseline variation and trophic shifts. By correcting for the possibility of baseline changes, unambiguous assignments of trophic position can therefore be made. Application this technique to a collection of *Bairdiella chrysoura* spanning multiple year classes was used to demonstrate the timing of ontogenetic diet shifts in this species. Juveniles (<80 mm standard length) were found to consistently occupy a lower trophic level than adults (>80 mm) based upon $\Delta^{15}N_{\text{glutamic acid-glycine}}$. Results obtained were compatible with previous estimates of ontogenetic effects on prey preferences derived from gut-content analysis.

Amino acid ^{15}N-based trophic determinations assume near-constant trophic fractionation in each compound, regardless of an organism's health or nutritional status. The fact that this fractionation is influenced by internal nitrogen processing within the organism argues against such consistency, however. Effects of condition on the magnitude of compound-specific fractionation and resulting trophic estimates were tested by comparing dry season and wet season samples of *Anchoa mitchilli* subject to seasonal starvation in the latter period from the Alafia River, Florida. Despite significantly lower measures of condition (as measured by length:weight ratios) in the wet season fish, no differences in amino acid fractionation were detectable between them. This suggests that amino acid-specific fractionation factors are in fact robust to

changes in organism health, although more rigorous assessment of this in culture is required.

Chapter 1: Introduction

Stable isotopic analysis of the major light elements of biological samples has been used for many years to advance the understanding of nutrient pathways, food webs, primary production patterns, animal migration, and a host of other subjects. Carbon isotopes are frequently used in studies of primary production and energy flow in ecosystems, since distinct patterns of isotopic fractionation are imparted by primary producers and are subsequently retained with only modest alteration during biological transfers (Smith and Epstein, 1971; Farquhar et al., 1989). Stable isotopes of nitrogen, in contrast, fractionate strongly during assimilation and therefore become characteristically enriched with increasing trophic position, thereby providing a means to map trophic transfers and food web structure (Deniro and Epstein, 1981; Minagawa and Wada, 1984; Peterson and Fry, 1987).

Typically, isotopic measurements of organic matter have been made by high temperature combustion of bulk samples to CO_2 and N_2 gases, the latter obtained via chemical reduction of nitrogen oxides. Automated techniques such as elemental analyzers linked to continuous-flow isotope ratio mass spectrometers make this process fast, reproducible, and logistically simple, leading to extensive applications in areas as diverse as ecology, biogeochemistry, archaeology, forensics, and product authentication

(Van Der Merwe, 1982; Peterson and Fry, 1987; Hobson, 1999; Hobson and Wassenaar, 1999; Bauer-Christoph et al., 2003; Slater, 2003)

A fundamental limitation of this technique, however, comes from the internal heterogeneity of the samples analyzed. Even the simplest biological materials typically are comprised of a wide range of complex molecules. All information on differences in isotopic compositions between different compounds is lost during the conversion of samples to simple anylate gases. While bulk isotopic analysis of organic samples reveals a great deal of useful data on their environmental histories, much potential information is also obscured by this process.

A means to address this shortcoming has been devised through compound-specific isotopic analysis (CSIA). This technique mates a separatory technology (typically gas or liquid chromatography) to a combustion interface and isotope ratio mass spectrometer to deconvolute complex mixtures, allowing sequential measurement of isotopic values of individual molecular species. While the necessity of online separations increases analytical time relative to bulk determinations, CSIA provides a much richer understanding of each individual sample, as values for a suite of compounds are obtained rather than a single measurement. It can utilize smaller sample sizes, and is generally less sensitive to contamination unless exogenous materials have the same molecular makeup as the sample itself. While having achieved broad acceptance in geochemical research, the applicability of this approach for addressing ecological problems in both modern and ancient systems has only recently been appreciated.

As the fundamental building blocks of proteins, amino acids are one of the most significant classes of nitrogen-bearing organic molecules and are a major constituent of most tissues. Structurally, amino acids are relatively simple, low-molecular weight compounds containing an amine group, a carboxyl group, and a side chain of varying composition. The side chain imparts chirality to all amino acids other than glycine (where the side chain is a single hydrogen atom), such that each exists as at least two isomers. Twenty amino acids, with a handful of posttranslational modifications, comprise virtually all known proteins.

The ubiquity and importance of these compounds suggest them to be a useful target for compound-specific isotopic analysis in investigations of biochemistry, trophic ecology, and nutrient cycling. Carbon skeletons of amino acids retain isotopic information about their biological origins, a fact that can be leveraged to gather data on sources of dietary protein and carbohydrates through comparison of essential and non-essential amino acids (Fantle et al. 1999; Jim et al. 2007). Nitrogen isotopes, while analytically more difficult to measure, provide a powerful resource for addressing questions of trophic ecology. The commonly cited trophic enrichment seen in bulk nitrogen isotope values is in fact the sum of fractionations occurring in individual amino acids, which are variable in magnitude based on different rates of nitrogen exchange through transamination reactions (McClelland and Montoya 2002; Chikaraishi et al. 2009). Functionally, this means that each sample produces a series of discrete measures spanning a continuum from unaltered source nitrogen values through compounds demonstrating very high levels of trophic fractionation.

In studies of both carbon and nitrogen isotopes of amino acids, one of the primary uses of CSIA relative to bulk measurements is in addressing the subject of context. Virtually all isotopic measurements used in ecological applications must deal with the problem of partitioning source- and process-related effects. Isotope values are typically used as tracers of nutrient or carbon flow, but they are also influenced by chemical conversion and differential uptake at various stages between primary source and ultimate sink. In practice, this means that bulk isotope measurements are highly sensitive to local conditions and therefore are of substantially less value when the environmental baseline underlying them is unknown. Unfortunately, this is frequently the case. It is often difficult to reconstruct the migratory history of highly mobile species, quantification of all nutrient sources into ecosystems is problematic, and the environmental histories of archaeological and paleontological samples are generally poorly known.

CSIA of amino acids, by providing a series of independent measures within a sample, can help resolve these difficulties. When the biochemical systematics leading to different compounds are understood, the patterns seen within each sample can provide environmental and ecological data independent of any external measures. This can include differences in $\delta^{13}C$ values between essential and non-essential amino acids (the former are highly conserved and must be derived from protein, while the latter integrate the entire carbon supply of an organism) or the ranges of nitrogen isotope values found between high-fractionating and non-fractionating amino acids (a subject that will be addressed extensively below). In either case, by parsing isotope data

normally combined in bulk samples into individual, discrete constituents, sample-specific insights can be obtained.

In order to address this subject fully, a review of the basic systematics of amino acid synthesis will be presented, along with reviews of existing literature on the application of CSIA to studies of both carbon and nitrogen isotopes of these compounds. Subsequently, the specific research objectives of this project will be addressed in detail.

1.1: Amino acid biochemistry

As noted previously, all proteins are comprised of 20 standard amino acids and their posttranslational derivatives. The most familiar classification scheme for these compounds in higher animals splits them into non-essential and essential categories. The former are readily synthesized from dietary carbon, while that latter have carbon skeletons that can only be manufactured by microbes, plants, and fungi, and must therefore be obtained from diet. This framework therefore provides a starting point for understanding internal carbon transactions and fractionation of ^{13}C.

A more precise understanding of carbon pathways and resultant isotopic fractionation must, however, address specific routes of amino acid biosynthesis. These pathways are complex and occasionally redundant, but are largely related to major cycles of cellular carbon turnover and energy production, specifically the citric acid cycle and glycolysis. A schematic of these pathways is shown in Figure 1.1.

Ten amino acids (six non-essential and four essential) are produced via modification of the citric acid cycle intermediates α-ketoglutarate and oxaloacetate. In the simplest of these reactions, α-ketoglutarate is combined with ammonia to produce glutamate (the anionic form of glutamic acid) by the action of the enzyme glutamate dehydrogenase (Solomons, 1988). Glutamate has a central role in biochemical nitrogen cycling. It participates freely in a class of reactions called transaminations, whereby an amino group is transferred from a donor amino acid to a suitable α-keto acid to form a different amino acid by the action of an appropriate transaminase enzyme.

Glutamate and α-ketoglutarate are participants (as either a nitrogen donor or acceptor) in the great majority of transamination reactions. Glutamate itself can also be modified to produce glutamine, arginine, and proline. Glutamine is formed by glutamine synthetase via the addition of an amide group at the carbon side chain terminus. It is a major carrier of bound ammonia in higher animals and an important component of nitrogen regulation and excretion cycles (Harvey and Ferrier, 2011). Proline is formed via cyclization of a glutamic acid semialdehyde intermediate. All five carbons are drawn directly from the original glutamate. Arginine derives from glutamate via a reversible pathway involving the urea cycle intermediates citrulline and ornithine.

Transamination of oxaloacetate generates the amino acid aspartate. Aspartate functions as a nitrogen transporter in the urea cycle, as well as being the parent compound for generation of asparagine, lysine, methionine, threonine, and isoleucine (Campell et al., 2005). Asparagine is formed via the simple addition of an amide group

from a glutamine donor, catalyzed by the enzyme asparagine synthetase. Lysine is synthesized from aspartate and pyruvate in bacteria and plants (the amide group is donated from a molecule of glutamate) while an alternate, complex pathway starting with α-ketoglutarate and acetyl-CoA is utilized by fungi.

Methionine and threonine are both products of homoserine, a four-carbon non-protein serine analog that cannot be synthesized by higher animals (explaining the essentiality of these amino acids) but that is itself a derivative of aspartate (Stryer, 1995). In methionine synthesis, homoserine combines with cysteine (the source of the thiol group) with subsequent cleavage of pyruvate to yield homocysteine. The latter molecule is then methylated at the thiol terminus, yielding methionine. The backbone of this molecule is derived from the aspartate, rather than the cysteine, carbon. While the carbon skeleton of cysteine (and therefore cystine) is derived from serine (see below), the conversion between homocysteine and methionine is readily reversible and provides a pathway for animals to generate cysteine using methionine as a sulfur source.

Threonine synthesis proceeds via phosphorylation of homoserine and subsequent hydrolysis (Hames and Hooper, 2005). While threonine has multiple stereocenters and therefore has four possible chiral configurations, only one of these (2*S*,3*R*-2-amino-3-hydroxybutanoic acid) is of biological significance in living cells (note that this is not the case in fossil material subject to diagenetic alteration). Threonine is converted to α-ketobutyric acid by threonine dehydrogenase in the first step of isoleucine synthesis. A

multi-step enzymatic pathway shared with valine (which is derived from an alternate α-keto acid, as described below) subsequently adds carbon from pyruvate and dehydrates the resulting molecule to the α-keto acid equivalent of isoleucine. The final step in this synthesis is transamination by glutamate.

The remaining amino acids derive their carbon skeletons from the breakdown or alteration of glucose. Six of these are direct products of glycolytic intermediaries. In the simplest of these reactions, transamination of pyruvate produces alanine. Like all transaminations, this is an equilibrium reaction and readily reversible. Alanine is produced in muscle tissue of higher organisms during anaerobic exercise and serves as an intermediary for transporting nitrogen and carbon to the liver (for excretion as urea and for energy production, respectively) (Smith et al. 1983). Along with glutamate and aspartate, it is therefore an important component of internal nitrogen cycles. Pyruvate also provides carbon for the synthesis of the essential amino acids valine and leucine (Stryer, 1995). The former utilizes an enzymatic pathway shared with threonine but beginning with pyruvic acid and ending with the transamination of α-ketoisovaleric acid. This compound also can undergo a condensation reaction with acetyl-coenzyme-A to generate α-isopropylmalic acid, from which subsequent modification and transamination produce leucine.

Oxidation of 3-phosphoglycerate and subsequent transamination forms the amino acid serine. The enzyme serine hydroxymethyltransferase then reversibly interconverts serine and glycine. Glycine can also be synthesized from CO_2 and NH_3 by glycine

synthase utilizing methylenetetrahydrofolate, but the actual occurrence of this reaction *in vivo* is questionable due to unfavorable reaction kinetics (Lehninger, 1975; Devlin, 2006). Cysteine and its dimerized form cystine are also derived from serine. In mammals, substitution of the hydroxyl group in serine with a sulfur atom from methionine creates cysteine. An alternate pathway in microorganisms catalyzed by cysteine synthase utilizes sulfide as a sulfur source.

Synthesis of the aromatic amino acids phenylalanine, tyrosine, and tryptophan begins with erythrose 4-phosphate (created by the pentose phosphate pathway, an alternate mechanism to glycolysis) and phosphoenolpyruvate (a product of oxaloacetate). A multistep enzymatic pathway leads to shikimic acid (an important progenitor for a number of biological aromatic compounds including lignin) and chorismic acid (Stryer, 1995). Further modification of the latter compound presents a branch point, with the action of chorismate mutase leading to phenylalanine and tyrosine (by transamination of phenylpyruvic acid and hydroxyphenylpyruvate, respectively). Tyrosine can also be formed by direct hydroxylation of phenylalanine in organisms not capable of de novo synthesis, a pathway that consumes the majority of dietary phenylalanine in higher organisms (Devlin, 2006). An extended pathway from chorismic acid leads to the creation of an indole side chain, which when substituted onto a molecule of serine produces tryptophan.

The final protein amino acid, histidine, utilizes ribose 5-phosphate from the pentose phosphate pathway as a precursor material. A very complex series of reactions leads to

a final molecule with carbon and nitrogen derived from ribose 5-phosphate, glutamate, and glutamine, with the purine ring donated from a molecule of adenine triphosphate (Lehninger, 1975).

The shared biosynthetic pathways impart similarities in certain groups of amino acids that are not intuitive based on structural considerations. Glycine and serine, for instance, are often quite similar in both $\delta^{13}C$ and $\delta^{15}N$ due to their rapid, reversible interconversion. Leucine and isoleucine, in contrast, are often distinct in $\delta^{13}C$ despite being structural isomers of one another since they are the products of widely divergent metabolic pathways.

Carbon isotopic compositions of amino acids in higher organisms are also known to be influenced by the ability of such organisms to synthesize the underlying α-keto acids. Non-essential amino acids, especially those deriving by simple pathways from major metabolic intermediates, will generally have $\delta^{13}C$ values representative of the aggregate carbon supply (protein, carbohydrate, and lipid) supporting an organism. In contrast, essential amino acids (as well as non-essential amino acids that incur a substantial metabolic cost to synthesize) are absorbed directly from dietary sources with minimal fractionation (Fantle et al., 1999). Variations in net dietary sources of protein and non-protein carbon can be clearly traced in this way (Jim et al. 2007).

Fractionation of $\delta^{15}N$ does not follow patterns of amino acid essentiality in a straightforward way. Rather, the rate of transamination appears to be the critical factor in determining trophic fractionation of nitrogen isotopes (Chikaraishi et al., 2007).

Amino acids participating in various within-organism biochemical nitrogen cycles (notably alanine, aspartic acid, and glutamic acid) in which transamination occurs frequently are imparted high levels of trophic fractionation, while those that are rarely transaminated retain a dietary signal. It is interesting to note that no transaminase enzymes exist for lysine and threonine, the latter of which commonly demonstrates a negative trophic fractionation. Phenylalanine- commonly used as the de facto indicator of source nitrogen due to its relatively easy chromatographic resolution- does not participate in transamination reactions to an appreciable extent (Campell et al., 2005). As previously noted, most phenylalanine incorporated from diet is transformed into tyrosine (itself a precursor for neurotransmitters and therefore often needed in excess of dietary supply), but no nitrogen isotopic fractionation is imparted to the residual material incorporated into proteins (Devlin 2006).

Finally, while transamination reactions are enzymatically possible for most essential amino acids, it is worth noting that they do not impart a "new" nitrogen signal to any appreciable extent to these compounds in higher organisms. While theoretically reversible, transamination reactions involving essential amino acids are functionally unidirectional due to the inability of the organism to synthesize the corresponding α-keto acid. While amino groups can be transferred from essential amino acids to α-ketoglutarate, the residual carbon skeleton, once catabolized, cannot be replaced. This means that nitrogen isotope fractionation in essential amino acids, if present at all, must be the result of catabolic processes.

1.2: Amino acid carbon isotope systematics

Investigations of the natural-abundance isotopic composition of carbon in protein amino acids have been undertaken for decades. This research has followed two main branches. Patterns of amino acid-specific fractionation in samples of modern organisms have been used to elucidate the processes of internal carbon cycling that underlie protein synthesis and metabolism. Early work in this area helped determine the mechanisms responsible for the bulk isotopic fractionations seen in photosynthesis and during trophic transfers, while more recent applications have examined differential dietary routings of proteins and carbohydrates in consumers. A related body of literature has used amino acid carbon isotopes as a tool for understanding the sources and preservational state of organic matter in fossils, and assessed the efficiency of these archives for paleoecological reconstructions.

The first analysis of amino acid carbon isotopes was done by Abelson and Hoering five decades ago, when the field of isotopic analysis was still in infancy (1961). The authors investigated incorporation of differing carbon sources into amino acids produced by photoautotrophic algae both by photosynthesis and by heterotrophy in dark-bottle cultures. The pattern of fractionation between amino acids was largely similar, while the extent of fractionation relative to carbon source was substantially different. This demonstrated that amino acid synthesis was decoupled from the photosynthetic process, utilizing photosynthate carbon and heterotrophically acquired carbon interchangeably. Instead, the authors surmised the central role of the citric acid

cycle in the formation of glutamic acid, from which many other amino acids were ultimately derived. This work, like many investigations in the subsequent three decades, relied on painstaking separation of amino acids from acid digests via ion-exchange chromatography followed by offline combustion of individual compound aliquots.

Further understanding of compound-specific fractionations during amino acid formation was provided by Macko (Macko et al. 1987). Several species of microorganisms were cultured on a variety of media of known isotopic compositions, and the resulting amino acid-specific fractionations were examined. There was a general increase in $\delta^{13}C$ values during incorporation of carbon into amino acids, with aspartic and glutamic acids exhibiting the greatest fractionations. This was thought to be due to the presence of multiple carboxyl groups in these molecules, the carbons of which are characteristically isotopically enriched relative to the remainder of the compound. Leucine, isoleucine, and lysine were relatively depleted, an effect ascribed to their shared formation pathway through acetyl coenzyme A (note that this does not account for the wide divergence in formation mechanisms between leucine and the latter two amino acids).

The first direct examination of dietary assimilation of amino acids came from a controlled feeding experiment where pigs were raised on pure C3 or C4-derived diets (Hare et al. 1991). Since these photosynthetic groups have differing characteristic fractionations for fixed carbon, the diets therefore had easily distinguishable isotopic signatures. Analysis of amino acids in bone collagen showed a nearly direct

incorporation of dietary glycine (despite this being a non-essential amino acid), serine, and threonine, while glutamic acid was found to fractionate heavily (+6-7 ‰). The important role of glutamate in the citric acid cycle and in amino acid biosynthesis and the resultant rapid turnover time for this molecule were assumed to explain this. Importantly, the pattern of amino-acid specific variation was found to be overlaid on top of the differences in the bulk dietary isotopic composition, suggesting that dietary signals propagated into tissue amino acids in a predictable and replicable way.

The application of online, compound-specific analysis of amino acid samples revolutionized this area of research when first performed in the early 1990s (Silfer et al. 1991). While amino acid quantitation had be performed via chemical derivatization and gas chromatographic separation for many years, several challenges needed to be addressed to adapt this method to isotopic analysis (Adams, 1974; Kaiser et al., 1974). Principal among these was the effect of carbon additions during derivatization to the ultimate isotopic compositions of the measured compounds. Derivatization schemes typically utilize reagents in quantities greatly exceeding analyate concentration. This was found to produce kinetic fractionations that prevented back-calculation of unknown amino acid values from mass balance with reagent compositions.

Fortunately, the magnitude of the kinetic fractionation was found to be highly reproducible when compounds were derivatized under identical conditions. It was therefore possible to derive amino acid-specific empirical correction factors that could be used as “effective” reagent isotopic values based on the results obtained from amino

acid standards of known isotopic composition. This mathematical technique underlies all subsequent GC-C-IRMS amino acid carbon isotope results.

Differences in trophic fractionation between essential and non-essential amino acids were investigated by Fantle et al. as part of a study of dietary sources supporting the blue crab, *Callinectes sapidus,* in mid-Atlantic estuaries (1999). Trophic fractionation of carbon isotopes was found to be greater in non-essential amino acids than in essential ones, suggesting that the latter were directly incorporated from diet with minimal alteration. Non-essential amino acids, in contrast, were subject to greater internal recycling and therefore displayed more deviation from dietary compositions. This study also touched on using amino acids to examine differences in protein carbon relative to whole-diet carbon. Essential amino acids were assumed to be characteristic of the protein component of diet, since their carbon skeletons could not be internally synthesized from other dietary constituents. Non-essential amino acids, which could be created de novo from dietary carbohydrates and lipids, were taken to represent carbon isotopic compositions of the entire integrated diet. The data presented here were one of the first applications of GC-C-IRMS analysis of amino acids and broke new ground in framing how the method could be useful in investigating carbon flow and trophic ecology. It is worth noting, however, that the values presented do not fully account for mathematical errors introduced in the correction for derivative carbon, and are quoted to precisions that are impossible to obtain with the techniques used.

A through treatment of error propagation and its effects on precision estimates in $\delta^{13}C$ measurements was provided shortly thereafter (Docherty et al., 2001). Analytical precisions for derivatized amino acid $\delta^{13}C$ values frequently equal the assumed instrumental precisions of GC-C-IRMS systems of approximately 0.3‰. Calculation of the isotopic value of the original, un-derivatized amino acids requires GC-C-IRMS measurements of both the unknown sample and a derivatized standard, while the standard itself must also be measured via EA-IRMS prior to derivatization. The errors in all three measurements are combined in the final result and are additionally weighted be the proportion of derivative to native carbon in the measured molecules. The authors demonstrated that high-carbon derivatization schemes are therefore inherently less precise that those that add minimal carbon, regardless of chromatographic performance gains obtained by their use. For common derivatization methods, maximum obtainable precisions varied from approximately 0.5 -1.5‰, based on the size of the carbon backbone in the amino acid and the number of functional groups requiring substitution.

The extent of interchange between dietary protein and carbohydrate carbon was tested by examining resource partitioning in nectar-feeding moths (O'Brien et al., 2002). As adults, moths in the genus *Amphion* feed exclusively on sugars in plant nectar. They therefore lack any protein component in their adult diet, and thus have no means of obtaining essential amino acids after metamorphosis. The authors used analysis of amino acid carbon isotopes to demonstrate that during egg production, essential amino acids used in reproduction were drawn from the pool obtained during the larval period

and carried an unaltered isotopic signal of the larval diet. In contrast, non-essential amino acids were synthesized from carbohydrates obtained in the adult diet, and were increasingly reflective of this carbon source with increasing age. A larger study of the same general design, utilizing a larger suite of butterfly and host plant species, verified these results shortly thereafter (O'Brien et al., 2005).

Building on the previous work of Hare et al., additional diet studies were conducted using pig bone collagen by Howland (Howland et al., 2003). Bulk collagen isotopic compositions showed considerable variation relative to diet $\delta^{13}C$ values based on the size and isotopic composition of the protein portion of the overall diet. The essential amino acids phenylalanine and leucine were incorporated directly from dietary protein without appreciable fractionation. Non-essential amino acids, as expected, largely tracked the isotopic composition of the total dietary carbon pool, with compound-specific offsets consistent between different dietary treatments.

Dietary routing of amino acids from diet into specific tissues was further investigated by controlled-diet studies in rats (Jim et al. 2007). Here, protein and non-protein dietary components with substantially different isotopic compositions were supplied and the resultant amino acid isotopic compositions of bone collagen were compared. As expected, essential amino acids were routed from diet to tissue with minimal isotopic fractionation. Mass balance calculations suggested that rather than being completely synthesized de novo, non-essential amino acids were also partially routed from dietary protein. The formulated diets in the study contained approximately 20% protein by

weight, a value in excess of the nutritional requirements of the test animals. The authors surmised that the extent of direct incorporation of non-essential dietary amino acids was directly related to this excess supply, thereby showing a mechanism by which information on dietary quality (i.e., protein content) might be gleaned from amino acid $\delta\ ^{13}C$ values.

Amino acid synthesis proceeds via different pathways in plants, fungi, and prokaryotes. The isotopic fractionation patterns that result from these pathways should therefore be distinct, and should be preserved in essential amino acids in organisms consuming producers of different types. Work on Antarctic microbial communities has shown that biosynthetic pathways impart specific isotopic fractionations in ^{13}C to various amino acids that form patterns unlike those of compounds resulting from abiotic synthesis (Scott et al. 2006). Further, these fractionation patterns were also shown to be distinct between carbon sources (fermenters, autotrophs, and methylotrophs/methanogens).

Efforts have also recently been made to use this fact to construct "^{13}C fingerprints" of various source organisms for essential amino acids (Larsen et al., 2009). C3 plants, fungi, and bacteria were cultured on amino acid-free growth media, and the resulting distributions of $\delta\ ^{13}C$ values in essential amino acids were compared. Linear discriminant analysis performed on this dataset classified all samples correctly with >99% confidence, and was also largely successful in associating insect samples in previous, external studies with their correct nutritional sources. Lysine, leucine, threonine, and valine were found

to possess the most diagnostic differences between taxa, suggesting they had the greatest divergence in biosynthetic mechanisms.

Organic inclusions in mineral matrices have long been recognized as a potential archive of paleoenvironmental data. The first reports of intact amino acid constituents in fossil materials were made more than half a century ago and were repeated confirmed shortly thereafter (Abelson 1954; Briggs 1961; Jones & Vallentine 1960; Swain & Kraemer 1969). Amino acids have also been shown to be commonly preserved in a variety of biominerals, such as bivalve shells, coral skeletons, and diatom frustules (Matter et al., 1969; Robbins and Brew, 1990; Sigman et al., 1999; Ingalls et al., 2003). Further investigation into diagenetic processes impacting these organic matter archives demonstrated that most complex molecules such as peptides are decomposed rapidly across geologic time, but that a portion of the original material may persist for millions of years (Akiyama and Wyckoff 1970). The rate and extent of diagenetic alteration vary with mineral structure, water content and temperature of the preservational environment, relative hydrophobicity of the organic compounds, and the nature of the association of the compounds with the mineral matrix (i.e., inter- vs intracrystalline), but in all cases remnant organic matter is substantially altered in composition from its original condition in all but the youngest samples (Robbins and Ostrom, 1995; Robbins et al., 2000; Sykes et al., 1995). While isotopic measurement of bulk organic residue extracted from fossils has been used to successfully deduce paleoecological data in a number of cases, analyses of this type are always confounded by the inability to

establish the preservational state and indigeneity of the material being examined (Iacumin, 2000; Bocherens et al., 1994; Ostrom et al., 1993).

Among the most predictable diagenetic processes is racemization of amino acid enantiomers. Amino acids (with the exception of glycine) are chiral molecules, possessing one or more stereocenters and two structural isomers (at minimum). Virtually all biologically produced amino acids (some bacterial production being the exception) are of the L-isomeric form. There is a natural thermodynamic interconversion between L- and D- isomers that eventually produces an equilibrium mixture of both forms with the passage of time. The rates of racemization are compound-specific and temperature dependent; if the mean temperature of the preservational environment can be estimated, it is therefore possible to use the extent of amino acid racemization as a dating tool (Bada and Protsch, 1973; Manley et al., 2000). The differences in racemization rate between compounds was also recognized as a tool with which to address the critical problem of establishing indigeniety of organic matter in fossils, since the extent of racemization of various amino acids in non-contaminated samples should follow a predictable pattern with age, even if the absolute concentration of them had changed substantially during diagenesis (Bada et al., 1999). Early methodologies of GC analysis of amino acid mixtures were therefore created in part to simplify enantiomeric determinations (Hartmut et al., 1977).

Enantiomeric separations as a means of assessing the indigeneity of organic matter were quickly expanded to include isotopic determinations. The first efforts in this regard

relied on time-consuming HPLC and ion exchange chromatography to resolve isomers of glutamic acid in a standard mixture and subsequently determined their isotopic compositions (Engel and Macko, 1984). While the technique presented utilized a greatly simplified sample relative to anything in the natural environment (a combination of the two enantiomers of a single amino acid), it did serve to demonstrate that the processes required for separation and isotopic determination of amino acids did not impart any isotopic fractionation to them.

An expansion of Engel's method to natural samples was performed shortly thereafter (Serban et al., 1988). Here, enantiomeric separations and isotopic measurements of glutamic acid were made on shell organic matrix separated from *Mercenaria* shells of Pleistocene age. Modest differences in isotopic composition were found between isomers (~4 ‰ in $\delta^{13}C$), however, it was unclear if this represented contamination with exogenous materials or was simply error caused in the sample preparation, which was quite complex. A follow-up study of land snails using similar techniques found that rigid adherence to cleaning protocols resulted in negligible measured fractionations between enantiomers in samples of similar age (Engel et al. 1994).

The ability to accurately determine the isotopic composition of amino acid constituents in biominerals without processing-related fractionation opened the door to investigations of the isotopic effects of diagenesis on residual material. Comparison of the isotopic composition of collagen amino acids from modern and fossil samples showed that Individual amino acids were consistently depleted or enriched relative to

bulk isotopic measurements regardless of sample age and despite variations in residual concentration (Tuross et al., 1988). Rather, changes in concentration of different amino acids (some of which are lost to diagenetic processes at substantially higher rates than others) could produce changes in bulk isotopic compositions over time far greater in magnitude than any breakdown-related fractionations of the individual compounds. Compound-specific measurements, therefore, should be more indicative of the original composition of the sample, especially in cases of questionable preservation.

The advent of GC-C-IRMS techniques for amino acid isotopic analysis greatly enhanced the efficiency of this area of study, as previously noted (Silfer et al. 1991). A combination of GC and LC analysis of amino acid enantiomers extracted from modern and fossil mollusk shells found small isotopic fractionations between isomers of acidic amino acids in both the fossil material and also modern samples where racemization had been induced by heating, suggesting that the assumption of fractionation-free racemization chemistry may not hold for this class of compounds (Silfer et al. 1994). Neutral amino acids, in contrast, showed no discernible difference in isotopic composition of enantiomers, suggesting these might provide a more effective means of assessing indigeneity. While the authors suggested a geochemical explanation for the results obtained, it is worth noting that the results for neutral amino acids were exclusively determined via GC-C-IRMS whereas the values for the acidic compounds came from LC separations, suggesting that methodological considerations may also have played a role. In any case, online GC separations were shown to produce comparable results to the offline separations they replaced.

Compound-specific amino acid analysis from bone collagen has also been used not just as a tool for correcting for variations in sample preservation, but as a source for additional information not available through bulk analysis. While bone collagen bulk isotopic compositions are frequently used for trophic determinations of archaeological samples, such measurements in areas of high aridity are known to be problematic (Schwarcz et al., 1999). $\delta^{13}C$ analysis of collagen amino acids from archaeological collections of human bones was used as a means of assessing marine protein consumption in arid coastal regions (Corr et al. 2005). The extent of fractionation between glycine (a non-essential amino acid) and phenylalanine (an essential amino acid) was found to be a strong indicator of marine protein consumption. The authors asserted the cause of this phenomenon to be differences in dietary carbon routing: phenylalanine was incorporated directly from marine protein sources, while glycine was largely biosythesized from an isotopically distinct pool of terrestrially-derived carbohydrate.

Analysis of environmental data from organic matrix of mollusk shells, while often suggested, has infrequently been accomplished. Robbins and Ostrom investigated the carbon isotopic composition of amino acids in shell matrix from *Polinices duplicatus* and *Mercenaria campechiensis* as part of efforts to understand differences in biochemical sources and diagenetic pathways of hydrophilic and hydrophobic soluble shell protein components (1995). They found substantial differences in the isotopic composition of compounds between these two categories, as well as evidence suggesting a migration of materials from the hydrophilic to the hydrophobic fraction during diagenesis. Further

research using the same species as well as *Strombus* spp. showed similar patterns present between hydrophilic and hydrophobic soluble compounds, and also demonstrated that bulk carbon isotopes in shell organic matter recorded differences in environmental conditions for comparisons using identical species harvested from different locations (Robbins et al. 2000).

One of the largest studies of this kind to date involved comparison of amino acid isotopic compositions of modern and fossil *Mercenaria* shells from a variety of habitats (O'Donnell et al., 2007). The authors found substantial variability in amino acid-specific ^{13}C fractionation between habitats (Virginia versus Florida) in modern shells, a fact they ascribed to phenotypic variation within the species but that clearly needs further attention, given the potential impact on interpretation of fossil records. There was also considerable variation in the isotopic composition of amino acids from different growth increments of the modern shells, but in this case the changes largely took the form of a shifting baseline with relatively constant amino-acid specific fractionations. This was taken as a sign of short term-environmental variability. Amino acid carbon isotopic compositions in modern samples typically spanned ranges of up to 25‰ within a single individual, with glycine showing characteristically enriched values and leucine generally being most depleted.

Fossil samples had similar patterns of amino acid $\delta^{13}C$, with samples showing roughly equivalent ranges of values (~25‰) and generally having those ranges bounded by the values of glycine and leucine. Amino acids recovered from fossils were enriched in $\delta^{13}C$

relative to modern samples, and the extent of this enrichment varied between compounds. Alanine, glycine, and glutamic acid showed the greatest relative enrichment. The authors concluded that while information on the ecology of fossil organisms could be recovered through compound-specific $\delta^{13}C$, fractionations as a result of diagenetic processes varied between compounds (although all tended towards enrichment of residual organic matter) and thus needed to be accounted for individually when reconstructing data from this archive.

Carbon isotopes of amino acids therefore provide a means of improving understanding of sources of primary production in modern systems, but appear to have the greatest utility in establishing the preservational state of fossil materials. This can be done by comparison of isotopic values of amino acid enantiomers, or by examining differences in the distribution of isotopes between compounds. By focusing studies on compounds that are relatively resistant to diagenetic alteration, a better understanding of the ecological context of ancient samples can also be achieved.

1.3: Amino acid nitrogen isotope systematics

The utility of amino acid nitrogen isotope measurements in investigating within-organism nutrient cycling has been recognized for many years. Early work in this area focused on sample preparation methodology with off-line, HPLC-based separations (Macko et al., 1986). These techniques allowed investigation of fractionation factors in transamination reactions at natural abundance, providing a framework for understanding molecular-level biochemical nitrogen transformations.

Inquiries into the relationships between nutrient sources and resultant amino acid isotopic compositions soon followed. Macko (1987) cultured bacteria and blue-green algae on a variety of inorganic nitrogen substrates as well as single-amino acid media and examined the resultant patterns of isotopic variability in extracted amino acids. Glutamic acid was generally highly enriched relative to other amino acids, fitting with its role as the main nitrogen "currency" in cellular nitrogen transformations via glutamate transamination. Other amino acids were more variable, suggesting organism-specific biochemical pathways.

The need to understand the effects of diagenesis on organic matter preserved in archaeological and paleontological samples led to early work on amino acid nitrogen isotopic compositions of bone collagen from higher organisms. Hare (1991) raised pigs on controlled diets and examined the resulting distribution of nitrogen isotopic values in bone collagen amino acids, as well analyzing and comparing collagen constituents from a suite of modern and fossil samples. All amino acids except threonine (an essential amino acid in animals) were found to be enriched in ^{15}N relative to diet, with the greatest enrichment occurring in glutamate-glutamic acid. This was again in keeping with the latter amino acid's central role in transamination processes as well as the urea cycle. Threonine, interestingly, was found to be sharply depleted relative to diet, with tissue-diet fractionation of approximately -5‰ in the culture experiment. The range of overall nitrogen isotope fractionation within a single organism was found to be substantial: threonine values from bone collagen of a variety of species were depleted by as much as 50‰ relative to glutamic acid from the same sample.

Development of GC-IRMS techniques greatly increased the utility of amino acid isotopic analysis. Gas chromatography- isotope ratio mass spectrometry (GC-IRMS) allows for automated, on-line separation of compounds, greatly increasing sample throughput and sharply reducing the amount of material required for analysis. Successful techniques for analysis of carbon isotopes preceded those for nitrogen by several years, owing to the lower concentrations of nitrogen in amino acids, the lower sensitivity of isotope ratio mass spectrometers for this element, and the relatively low amounts of the high-mass isotope generally present.

The methodology employed in this technique is similar to that for carbon isotopic measurements via GC-IRMS. Compounds are separated on a chromatographic column then passed into an inline combustion reactor where oxygen is supplied via metal oxides held at temperatures from 850-1100^{o} C. Organic molecules are converted into CO_2, NO_x, and water, which are then swept by the carrier gas stream towards the mass spectrometer. For nitrogen analysis, it is essential that NO_x be quantitatively reduced to N_2 gas prior to analysis, so a reduction reactor of high-temperature metallic copper is commonly plumbed in-line after the combustion reactor. Additionally, CO_2 must be quantitatively separated from the gas stream before entering the ion source of the mass spectrometer to prevent interference from CO fragments, which occupy the equivalent range of masses to N_2. Typically, this is done with a cold trap made by passing a coiled section of transfer capillary through a dewar of liquid nitrogen. This can also serve to remove water in the absence of a dedicated water trap, although this will shorten the time the system can be used before the capillary is blocked by ice formation.

The first successful report of GC-IRMS measurements of amino acid nitrogen isotopes was made in 1994. In this study, a series of amino acid standards of known isotopic composition were derivatized to n-acetyl propyl esters and analyzed at low-nanomole concentrations. Values obtained were precise to within 0.4‰ and showed no consistent isotopic offsets to offline-combusted samples when quantities of amino acids injected were greater than two nanomoles (Merritt and Hayes, 1994).

As with early offline work, much of the focus of this research was procedural in nature. The researchers tested a variety of system configurations, paying particular care to the effects of combustion and reduction reactor design. The choice of metal used to form oxides in combustion reactors impacts the operation of these systems in a variety of ways, principally by varying optimal operating temperature, resultant free oxygen bleed, and total oxygen-donating capacity prior to reactor recharge. Typically, reactors are constructed using copper, nickel, or a combination of these and additional catalysts. Here, the efficiency of copper- and nickel-based reactors was directly compared. Both yielded acceptable performance, although nickel reactors had greater capacity for supplying reactive oxygen, less production of free oxygen at operating temperature, and longer service periods before reoxidation. The authors also noted that nickel reactors facilitated both combustion and reduction, without the requirement for a separate reduction reactor. The attention paid to methodological concerns in this study laid the groundwork for practical applications to follow.

Application of this technique to biological samples (rather than laboratory standards) followed several years later. Metges et al. used n-acetyl propyl esters and n-pivaloyl propyl esters to investigate synthesis of essential amino acids by microflora in the gut of pigs (Metges et al., 1996). This study quantified variability in analytical results between different sample preparation techniques based upon resulting differences in chromatographic resolution. The experimental data, however, were based upon artificial enrichment of gut ^{15}N content far above levels of natural abundance. The essential amino acid lysine, which must be synthesized by microorganisms and which does not participate in transamination reactions in higher animals, was found to be synthesized within the gut and incorporated into body tissues through the use of isotopically labeled urea and ammonium chloride.

Natural-abundance measurements were made shortly thereafter by the same group in a study of free amino acids present in human blood (Metges and Petzke, 1997). Threonine and phenylalanine were shown to be consistently depleted in ^{15}N, while alanine, proline, and leucine, and ornithine (a non-protein amino acid central to the mammalian urea cycle) were enriched. For subjects on natural diets, glutamic acid was found to be highly variable (presumably because of differences in individual diets) and to have maximum enrichments substantially greater than other compounds. Subjects on a single, controlled diet showed comparatively lower levels of variation.

A seminal study of amino acid-specific nitrogen isotopic relationships between marine primary producers and consumers was performed by McClelland and Montoya

(2002). The authors found that fractionation patterns varied substantially between different amino acids, and defined two broad groupings reflecting this behavior. "Source" amino acids (glycine, lysine, phenylalanine, serine, threonine, and tyrosine) showed negligible trophic fractionation, and therefore recorded the nitrogen isotopic baseline of the system in which an organism existed, while "trophic" amino acids (alanine, aspartic acid, glutamic acid, leucine, isoleucine, proline, and valine) fractionated strongly up food chains, and therefore were taken to be indicative of trophic position.

The processes driving these patterns were not fully elucidated, but the authors surmised that the extent of trophic fractionation was related to the rate at which amino acids participated in transamination and deamination reactions. Amino acids in which the amine group was highly conserved did not fractionate, while those that rapidly exchanged amine groups fractionated sharply. The high degree of trophic fractionation observed in glutamic acid (~7‰) was seen as indicative of this process, since glutamic acid is a central component of biochemical nitrogen cycles via glutamine and α-ketoglutarate pathways and a precursor material for the synthesis of many other amino acids. It was also observed that the distribution of compounds between source and trophic categories did not match well with the classic essential and nonessential groupings, which are based upon the capacity to synthesize amino acid carbon backbones (as opposed to amine groups).

The differential behavior of the two suites of compounds within an organism suggested two applications beyond the scope of what conventional isotopic analysis could provide. First, the large degree of fractionation in the trophic amino acids was asserted to provide a greater range for assigning fractional trophic positions, although this did not fully account for the lower precision of these measurements relative to bulk isotopic analysis. Second, and more significantly, the presence of compounds recording the nitrogen isotope baseline for the ecosystem from which an organism was drawn eliminated the need to infer this value from analysis of co-located primary producers or consumers. This is a requirement for the interpretation of bulk isotope values and is commonly held to be a weakness of the technique (Post, 2002; Casey and Post, 2011).

Internal encoding of isotopic baselines therefore enables greater understanding of samples for which the environmental context is not clearly understood. Long-distance migrant fish capable of using widely disparate feeding habitats fall into this category: isotopic baselines of marine food chains can change over short spatial and temporal scales, and it is generally not known with certainty where such animals have been located (and presumably have fed) over their lifetimes. Marine plankton can also benefit from analysis in this way, since nutrient parameters can change over short spatial and temporal scales and such changes are rapidly incorporated into them due to their small size and fast turnover times. Fossilized or archived samples of any kind likewise are good candidates, since commonly the environmental context for such samples is not fully understood.

The first application of this technique focused examining the role of N_2 fixation in providing fixed nitrogen to pelagic marine zooplankton (McClelland et al., 2003). This study linked changes in bulk $\delta^{15}N$ values of zooplankton across a transect of the Atlantic Ocean to spatial variation in nitrogen fixation by the marine cyanobacterium *Trichodesmium.* Both source and trophic amino acid values followed the gradient seen in the bulk data, while the offset between them remained essentially constant. This demonstrated that the bulk changes were driven by a changing ecosystem baseline (due to variable input of ^{15}N-depleted organic nitrogen via fixation) rather than a change in the trophic position of the zooplankton.

Knowledge of amino acid-specific nitrogen isotopic compositions can also assist in understanding observed patterns of variation in bulk isotopic compositions within a single species. Schmidt et al. determined amino acid nitrogen isotopic values for tissue samples from *Euphausia superba* in an attempt to resolve sex-specific differences in bulk ^{15}N values that did not appear to be trophically related (2004). As with the previous work by McClelland and Montoya, Phenylalanine, serine, threonine, and glycine tended to be relatively depleted and glutamic acid was highly enriched in ^{15}N. Interestingly, aspartic acid was found to vary sharply between males and females in this study (~ 6‰). This high degree of offset, combined with the fact that aspartic acid was a major constituent of euphausid protein, was taken to explain the differences seen in the bulk data. The fact that other amino acids were similar in both sexes showed that biochemical effects (likely resource partitioning related to reproduction) were the ultimate drivers of the bulk ^{15}N values rather than changes in diet.

In addition to work on low-level planktonic consumers, CSIA of amino acid nitrogen has also been applied to a study of the trophic position of top marine predators, specifically Yellowfin Tuna (*Thunnus albacares*) in the eastern tropical Pacific (Popp et al. 2007). Variation in bulk nitrogen isotopic composition of individuals taken in different regions during this study indicated either a regional shift in ecosystem baseline or a habitat-related change in trophic position of sampled fish. To resolve this question, amino acid samples were analyzed for fish collected from 8^{o} S to 24^{o} N latitude in the eastern Pacific along a gradient of increasing bulk nitrogen isotopic values.

The authors reasoned that if a change in trophic position accounted for the enriched values in the more northerly specimens, then the trophic-sensitive amino acids would show a similar trend, while the source-recording amino acids would show no change. In contrast, a change in ecosystem baseline should be reflected uniformly in all amino acids (similar to what was found by McClelland et al 2004). The data clearly supported the latter case, showing that the ambiguous bulk-level isotopic enrichment was, in fact, the product of differing regional nitrogen isotope baselines. In so doing, this verified that the ability to parse source vs. trophic effects in nitrogen isotopes was preserved even at trophic levels substantially removed from primary producers.

Consideration was also given to the most effective means of quantifying trophic position based on this technique. Differing methodologies for sample preparation (specifically, differences in amino acid derivatization scheme and GC column chemistry) change the resulting distribution of isotope data obtained from this type of analysis.

Amino acids that are well-resolved by one particular methodology can therefore be compromised or absent in others. While McClelland and Montoya had suggested using phenylalanine (source) and glutamic acid (trophic) as representative compounds for trophic level assignments, the methodology of this study suggested greater robustness in phenylalanine-glycine comparisons. More significantly, the authors showed that taking the difference of overall trophic and source average values as a composite index produced results not significantly different from that obtained from individual amino acid pairs.

The large fractionation of trophic amino acids up food chains (~7‰ for glutamic acid) has been suggested as a means for increasing accuracy in nitrogen isotope-based trophic determinations. Chikaraishi et al. determined amino acid nitrogen isotopic compositions for a suite of macroalgal species and associated gastropod mollusk grazers (2007). A similar pattern of source vs. trophic amino acids was observed here, but the focus was on differences in trophic estimates using bulk vs. compound-specific methods. Gastropods fractionate somewhat less that the canonical 3‰ per trophic level in bulk nitrogen isotopes. Comparisons using the fractionation of glutamic acid alone between producers and consumers found fractionation factors for this individual compound that were consistent with previous reports (~7.6 ‰ for a single trophic level increase), despite bulk $\Delta^{15}N_{producer\text{-}consumer}$ values of only 0.5 - 2.0. The authors also went into further detail describing the mechanisms driving amino acid nitrogen systematics, emphasizing the role of the role of deamination in maintaining nitrogen balance in

heterotrophs and describing the likely outcome of this process in terms of isotopic fractionation.

Nitrogen balance, and the possibility of synthesizing non-essential amino acids with nitrogen from essential ones under conditions of nutrient limitation, has also been investigated using amino acid CSIA (Loick et al., 2007). In this study, the degree of correlation of $\delta^{15}N$ values of leucine (an essential amino acid) with those of a suite of nonessential amino acids was used as a proxy for transfer of nitrogen from essential to non-essential amino acids via deamination and transamination pathways. Leucine values were shown to be more strongly correlated with alanine, aspartic and glutamic acids, proline, and tyrosine at sites of nitrogen fixation (which were assumed to be nitrogen-limited) relative to sites with abundance inorganic nitrogen supplies.

The extent of preservation of 15N patterning in amino acids in marine detrital pathways was researched by McCarthy et al. (2007). Here, the nitrogen isotopic composition of amino acids in algae, sinking particulate organic matter, and dissolved organic matter were compared. The distribution of values found in living cells was similar to that reported in previous studies, although glycine and proline were both notably depleted in wild samples relative to previous laboratory cultures. Source-trophic offsets (here measured by using differences of mean values for each amino acid group rather than using individual diagnostic compounds) were consistent with primary producer signatures. The values in shallow-water sinking POM (~100M) were found to have a larger mean source-trophic offset (likely due to the POM not being entirely

derived from primary producers) than did the algal samples, but the patterns of offsets between different amino acids were similar. In contrast, deep-water (1000-3500M) sediment trap samples showed variable behavior in the isotopic composition of alanine, isoleucine, threonine, and phenylalanine, which the authors took to be indicative of bacterial heterotrophy and amino acid resynthesis.

The reliability of different source-trophic offset schemes for determining organism trophic level was investigated by Chikaraishi et al. (2009). The authors established a simple mathematical formula for amino acid trophic level determination and then tested the suitability of different source-trophic pairs as indicators. Trophic level was characterized as:

$$TL_{x/y} = (\delta^{15}N_x - \delta^{15}N_y - \beta_{x/y})/(\Delta_x - \Delta_y) + 1$$

Where x and y are the selected trophic and source amino acids, respectively, $\beta_{x/y}$ gives the offset between these two amino acids in primary producers, and Δ gives the trophic fractionation for a given amino acid. The glutamic acid – phenylalanine pairing was found to provide the most reliable results based on a review of available published data and on new results with cultured zooplankton and fish. Both glycine and serine were found to be variable in their extent of trophic fractionation and therefore were not considered suitable source indicators, while proline and leucine were the most variable trophic indicators.

While conceptually elegant, the numbers obtained for β and Δ were drawn from a relatively small pool of available data (approximately 30 published values) and may be

biased based on differences in chromatographic performance of differing derivative types. Further refinement of this technique will require additional data and better understanding of the underlying mechanisms that cause amino acid- specific fractionation.

The potential difficulties with using any individual trophic-source pairs were addressed in a study of zooplankton trophic position in the tropical Pacific (Hannides et al. 2009). The authors found that amino acid-derived trophic positions of primary and secondary consumers in zooplankton samples did not change significantly over a ten year sampling period, despite substantial variability in isotopic baseline over this time period. While trophic estimates were made based on the glutamic acid-phenylalanine pairing, the utility of using source-trophic averages was also tested. Alanine, aspartic acid, and glutamic acid (dubbed “trophic group II” amino acids) were found to have the most consistent levels of trophic enrichment. Trophic determinations made using averaged trophic-II amino acids and the source amino acids serine, glycine, and lysine did not differ significantly from glu-phe values, in contrast to the results in the study by Chikaraishi. This study also noted that results generated from formalin-preserved zooplankton samples did not differ significantly from fresh samples, suggesting that a large volume of archived samples may be amenable to trophic determination via the amino acid technique.

The majority of work done making trophic determinations based on amino acid nitrogen values has focused on marine species. There is evidence that source-trophic

patterning in amino acid isotopic compositions can be generalized to higher organisms as well, however. A study of three Antarctic penguin species found that accurate trophic determinations could be made in birds using the glu-phe technique, and that differences in nutrient sources could be observed between species with differing foraging habitats (Lorrain et al., 2009). Admittedly, these animals derived all of their nutrition from marine sources, but this work suggested their internal physiological processing of nitrogen appears to follow a similar paradigm to that in marine organisms.

The most recent applications of amino acid nitrogen isotopes have begun to move from present-day marine ecology into paleoecological reconstructions. Capitalizing on existing work utilizing amino acid carbon isotopes in bone collagen, Styring examined nitrogen isotopic compositions from preserved collagen in marine and terrestrial animal skeletons of Holocene age as well from human skeletons from the same age and region (Styring et al., 2010). In marine samples (seal and whale), the typical pattern of source-trophic fractionation was observed, suggesting that protein inclusions in biominerals can preserve biological signatures of their parent organisms. In contrast, terrestrial species deviated sharply from expected behaviors, with ungulates showing essentially no glu-phe offsets and with tortoises (which can excrete nitrogen as uric acid and may manage internal nitrogen cycling differently) having glutamic acid values significantly depleted relative to phenylalanine. The latter did not vary significantly between all organisms from the same region, which was taken to signify similar nitrogen sources- a proposition which seems suspect given the differing habitats involved. Threonine, as reported in some earlier studies, was increasingly depleted with trophic level. This was true for both

marine and terrestrial collagen samples. Unfortunately, all of the terrestrial animals in this study were primary consumers, while the human population examined utilized a mixed marine-terrestrial diet. Clearly, better understanding of glutamic acid-phenylalanine fractionation (and source-trophic fractionation more generally) during trophic transfers in terrestrial species is urgently needed if amino acid trophic determinations are to be taken out of the marine realm.

In another very recent paleoecological study, isotopic nitrogen isotopes of growth bands sectioned from the annual growth bands of deep-sea gorgonians (*Primnoa resedaformis*) were used to reconstruct changes in nutrient supply in the western North Atlantic (Sherwood et al., 2011). Amino acid nitrogen isotopic analysis performed on a subset of these samples was used to correct for any potential changes in trophic position over the course of the study. No trophic variation was observed, leading the authors to conclude that recent changes in bulk nitrogen isotopic compositions in the gorgonian tissue were indicative of changes in source water partitioning in the overlying water column. Specifically, a ~1 per mil depletion in bulk $\delta^{15}N$ during the past 30 years was attributed to increased presence of warm, nutrient-rich subtropical waters driven by fluctuations of the North Atlantic Oscillation.

A synthesis of this existing research leads to several recognizable main points:

There is a consistent pattern of fractionation in amino acid nitrogen isotopes across trophic steps. Certain amino acids that are major metabolic intermediaries and their direct derivatives (notably glutamic and aspartic acid and alanine) fractionate sharply up

food chains. Conversely, other amino acids (phenylalanine, lysine, glycine, serine, and methionine) fractionate modestly or negligibly, preserving a record of ecosystem nitrogen baseline values. The amino acid threonine appears to exhibit a strong negative fractionation during trophic transfers.

The offsets between source (non-fractionating) and trophic (high-fractionating) amino acids provides an internal encoding of organism trophic position, without the requirement for ecological context. Source amino acids reliably track changes in ecosystem baseline nitrogen supporting an organism in both space and time.

Various formulas for rigorous assignment of trophic level have been conceived, but there is no firm agreement on which of these is best. Where chromatographic performance allows, the most common measure of this type utilizes the difference in $\delta^{15}N$ between glutamic acid and phenylalanine. Mean differences between suites of source and trophic amino acids have also been proposed, and may be less vulnerable to methodological differences in the determination of individual compounds.

The great majority of amino acid nitrogen isotope research in trophic ecology has focused on the present day marine environment. What data are available from terrestrial species suggest that the robust relationships seen in the marine environment may be more complicated or be absent from terrestrial ecosystems. Further research is needed in this regard.

1.4: Research objectives

Compound-specific isotopic analysis of amino acids is still a fairly novel research method, and there are still areas of considerable uncertainty in the interpretation of the resulting data. This study aims to address a number of these uncertainties, with a focus on applications in areas of interpretation of biomineral archives and on questions of trophic ecology.

CSIA is a complex and demanding analytical technique, and its application to studies of amino acids requires further specialization. As such, a thorough review of the methodology and instrumentation involved will be presented in the following chapter, prior to the presentation of individual research questions. This will include discussion of the chemical methods required to separate amino acids from bulk tissue samples, the means used to purify and derivatize them for GC separation, and the design of the overall GC-combustion-mass spec system used for the analysis.

Subsequently, the following specific research topics will be addressed:

Organic matter preserved in shells is often cited as a potential archive of paleoenvironmental data due to its slow rate of decomposition (Sykes et al., 1995; Bada et al., 1999). The isotopic composition of amino acids in this material provides an exceptional opportunity for gathering data about the parent organism's environment, as it should reflect the same biochemical processes involved in soft tissue formation and also provides a means to assess the extent of diagenetic alteration. Existing analyses have focused on differences between species or widely separated populations (Robbins,

Andrews, and Ostrom 2000; O'Donnell, Macko, and Wehmiller 2007). Whether this archive effectively retains information on spatial scales relevant to the study of local environmental gradients is unknown. Samples of *Crassostrea virginica* tissue and shell organic matter collected along an estuarine salinity gradient in Rookery Bay, FL, will be used to test the fidelity of amino acid isotope values between organic matter pools (tissue vs. shell) and will also examine how bulk isotopic changes are preserved in individual compounds.

Shell organic matter is a complex mixture of aqueous-soluble and insoluble biomolecules, the proportions of which vary sharply between species (Robbins et al., 2000). For purposes of isotopic analysis, it is frequently treated as uniform or is studied via an easily captured subset (i.e., conchiolin) of the overall material present. Interspecies comparisons using bulk isotopic values obtained in this way are confounded by compositional differences in shell organics between organisms, which manifest themselves as species-specific offsets in such values between body tissues and shell matrix proteins. Compound-specific amino acid nitrogen isotopic analysis will be used to compare shell organic matrix composition between a suite of mollusk species collected in St. Joe Bay, Florida, that spanned multiple trophic levels, and the results obtained will be compared to known trophic relationships as well as corresponding isotopic values from body tissues.

Amino acid nitrogen isotopes have been used to show changes in ecosystem baseline values within single species, and have also shown consistent fractionation patterns

between organisms occupying different trophic levels (McClelland and Montoya 2002; Chikaraishi et al. 2007; Hannides et al. 2009). These results strongly suggest that compound-specific nitrogen isotopic analysis of amino acids should provide a means of detecting ontogenetic trophic shifts within individual species, but there are not yet any published records showing such changes. Such growth-related diet shifts are often important determinants of recruitment success in fish species, so this seems to be a potential application of amino acid CSIA with great utility. A collection of silver perch (*Bairdiella chrysoura*) containing both juvenile and adult individuals from Tampa Bay, FL will be used to demonstrate the feasibility of detecting growth-related trophic changes with this method. This species is endemic and well-studied in the region, and information on diet composition based on previous gut content analysis will be compared to the isotopic results obtained here.

Trophic determinations based on amino acid nitrogen rely on constant fractionation factors between amino acids within an organism, regardless of physiological state (Chikaraishi et al. 2009). The sensitivity of these values to changes in organism condition is unknown, however. Bay anchovies (*Anchoa mitchilli*) in the Alafia River, Florida, experience seasonal starvation during the summer months due to stratification induced by high levels of precipitation, and therefore present a means of testing the robustness of amino acid nitrogen isotopic distributions to changes in condition. Amino acid nitrogen isotopic compositions of specimens collected during dry (May 2011) and wet (July 2011) seasons will be compared and indexed to individual condition estimates.

Finally, a synthesis of the main ideas that can be generalized from the specific research questions will be provided to summarize the findings presented here, to outline limitations of this technique, and to address future areas of needed research.

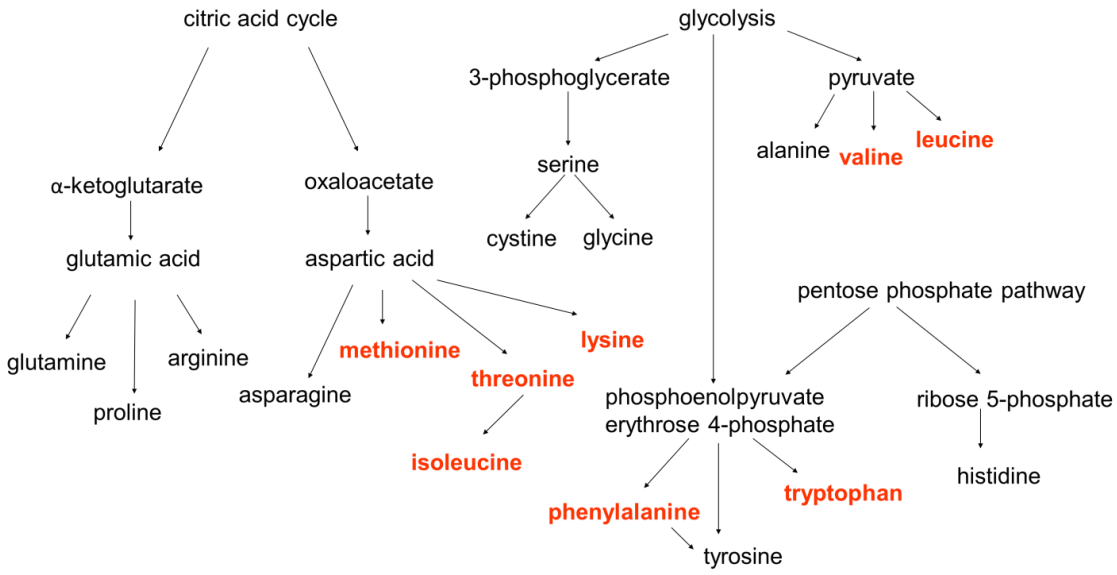


Figure 1.1: Generalized model of amino acid biosynthetic pathways. Boldface type indicates amino acids that are essential in higher animals. Modified from Hames and Hooper(2005).

Chapter 2: Equipment & analytical methods

2.1: Amino acid derivatization considerations

Amino acids are not naturally amenable to gas chromatographic separation due to their low volatility and large number of functional groups (Silfer et al. 1991). Compound-specific isotopic studies of amino acids via GC-C-IRMS therefore require that they be derivatized to more suitable forms prior to analysis. This generally involves substitution reactions at the carboxyl and amino terminuses of the molecule, along with any hydroxyl, amino, or thiol groups on the side chain.

Derivatization techniques used in GC-C-IRMS studies of amino acids are almost uniformly drawn from pre-existing efforts at GC-MS analysis of these compounds (Kaiser et al., 1974; Adams, 1974; Sobolevsky et al., 2003; Suresh Babu et al., 2005). GC-MS systems operate under different constraints from IRMS systems. Specifically, they can utilize much more dilute samples and have much better ability to deal with overlapping peak areas. Many of these derivatives are therefore imperfect for GC-C-IRMS usage. The most commonly used of these methods are outlined below.

Silylation is a very popular single-step derivatization for amino acid GC-MS analysis. It involves substitution of functional groups with various silyl groups (most often trimethylsilyl, $(CH_3)_3Si$-). These reactions are typically single-step and can utilize mild

temperature and pH conditions. Unfortunately, several shortcomings specific to GC-C-IRMS make this method unsuitable for CSIA. The silicon present in the silyl groups is thought to form complexes with the oxidation catalysts present in the GCC combustion reactor, shortening reactor life (Meier-Augenstein, 2004). There are also reports of damage to common chromatographic column stationary phases when loaded with concentrations of these derivatives required for isotopic analysis (Hofmann et al., 2003). The amount of carbon that can be added via silyation is quite large (the popular ter-butyl, dimethyl silyl group contains 6 carbons), potentially temporarily overloading the oxygen-donating capacity of the combustion reactor and resulting in CO formation and non-quantitative combustion (Merritt et al., 1995). This is especially problematic in measurements of $\delta^{15}N$ due to the much larger quantity of analyate required. Also, the large amount of reagent carbon relative to the native amino acid carbon also reduces the precision obtainable in measurements of $\delta^{13}C$ even when quantitative combustion is maintained.

N-pivaloyl propyl esters (NPIP) have seen use in CSIA, especially in applications focused on amino acid $\delta^{15}N$, due to their excellent chromatographic resolution (Metges et al., 1996; Metges and Petzke, 1997; Metges and Daenzer, 2000; Petzke et al., 2005). In this technique, the carboxyl terminus of each amino acid is esterified with acid propanol, while the amino terminus is protected with a pivaloyl group ($H_9C_5O_2$-). As is the case with silyl-based derivatives, this technique adds substantial quantities of carbon to each amino acid molecule. The carries similar risks of reactor overload and reduced $\delta^{13}C$ precision.

Acylation of the amino group using trifluoroacetic anhydride and esterification of the carboxylic acid group with acidified alcohols produces trifluroacetic esters (TFA esters). Like NPIP derivatives, these compounds show excellent chromatographic resolution on a variety of GC stationary phases, and are therefore widely employed (Silfer et al. 1991; Engel et al. 1994; Macko et al. 1997; McClelland and Montoya 2002). They also have a more favorable ratio of reagent:analyate carbon relative to the previously described techniques, increasing maximum obtainable precisions for measurements of $\delta^{13}C$. The presence of fluorine in the derivatizing reagents is problematic, however, as it forms metal halide complexes in the combustion reactor, irreversibly poisoning the catalysts and depleting the capacity of the metals to bind oxygen. Combustion of fluoridated derivatives is also thought to produce hydrofluoric acid which can attack downstream instrument components.

Acyl esters of amino acids are made by esterifying the carboxyl group with an acidified alcohol and subsequently acylating the amino group with acetic anhydride. The most commonly used of these are n-acetyl isopropyl esters, which were used in the first successful study of amino acid $\delta^{15}N$ via CSIA (Merritt and Hayes, 1994). They have a similar reagent:analyate carbon ratio to TFA derivatives but do not share the latter's problems with system damage. Despite being relatively simple to produce and having reasonably long shelf lives, they have been infrequently used due to their relatively poor chromatographic performance on common low-polarity stationary GC phases (Hofmann et al., 2003). On high-polarity columns (notably Rtx 233 and OV 1701) this difficulty can

be overcome, however, with separations equivalent to those of TFA esters obtainable (Meier-Augenstein, 2004; Corr et al., 2007b).

A recently developed alternative to n-acetyl isopropyl esters substitutes methanol in the esterification reaction to produce n-acetyl methyl esters (Corr et al., 2007a). These add only a minimal number of carbons to the original amino acid skeletons, and therefore provide a means to improve maximum obtainable precision for measurements of $\delta^{13}C$. They are considerably more volatile than other derivatives, however, and therefore are less reliable to produce, often being lost in evaporative removal of solvents.

2.2: Amino acid $\delta^{13}C$ calculations

$\delta^{13}C$ values measured for derivatized amino acids are the product of both the carbon native to the molecule as well as that contributed by the derivatizing reagents used in sample preparation. The derivatizing reagents used in these reactions are present in quantities greatly exceeding stoichiometric balance, leading to kinetic fractionation and deviations in the isotopic compositions of the products away from those predicted by simple mass-balance models. The extent of this fractionation varies between compounds, but has been shown to be stable and reproducible regardless of initial isotopic composition and concentration for each individual amino acid, so long as reaction conditions are held constant (Silfer et al., 1991). This allows for the calculation of "effective" isotopic values for the reagents used by derivatizing and measuring standards of known isotopic composition:

$$\delta^{13}C_{reagent} = \frac{\left(\delta^{13}C_{A.A.derivative} - (X * \delta^{13}C_{A.A.})\right)}{1 - X}$$

Where $\delta^{13}C_{A.A.derivative}$ is the measured value of the derivatized amino acid, $\delta^{13}C_{A.A}$ is the value of the underivatized standard amino acid, and X is the proportion of the total carbon in the derivative derived from the original molecule. This proportion varies based on the number of carbon atoms in the amino acid and the number of functional groups requiring derivatization (Table 2.1). Rearranging terms and plugging in the calculated reagent isotopic values allows for the determination of the amino acid carbon isotopic composition of unknown samples:

$$\delta^{13}C_{A.A.} = \frac{\left(\delta^{13}C_{A.A.derivative} - \left((1 - X) * \delta^{13}C_{reagent.}\right)\right)}{X}$$

2.3: Propagation of error in $\delta^{13}C$ corrections

An important outcome from the corrections required to obtain the carbon isotopic compositions of amino acids in this way is the increase in uncertainty of the resultant values. For any value obtained as a function of multiple measurements, the overall error is given by:

$$\sigma^2{}_{F(a,b,c)} = \sigma^2{}_a \left(\frac{\partial F}{\partial a}\right)^2 + \sigma^2{}_b \left(\frac{\partial F}{\partial b}\right)^2 + \sigma^2{}_c \left(\frac{\partial F}{\partial c}\right)^2$$

This can be rearranged in the case of amino acid carbon isotopic values obtained from measurements of derivatives as follows (Docherty et al., 2001):

$$\sigma^2{}_C = \sigma^2{}_S \left(\frac{n_S}{n_C}\right)^2 + \sigma^2{}_{SD} \left(\frac{n_S + n_D}{n_C}\right)^2 + \sigma^2{}_{CD} \left(\frac{n_C + n_D}{n_C}\right)^2$$

where C = the compound of interest

S = the standard used

D = derivatizing agents

SD = derivatized standard

CD = derivatized compound

n = the number of carbon atoms in each constituent

Since each unknown compound is corrected using a known standard of the same molecule, this can be simplified to:

$$\sigma^2{}_c = \sigma^2{}_S + \sigma^2{}_{SD} \left(\frac{n_{SD}}{n_C}\right)^2 + \sigma^2{}_{CD} \left(\frac{n_{CD}}{n_C}\right)^2$$

The variance contributions from the measurement of both the standard (σ^2_{SD}) and the sample (σ^2_{CD}) increase as the carbon contribution by the reagents increases. The maximum obtainable precision of compound-specific amino acid $\delta^{13}C$ values is therefore heavily influenced by the derivatizing reagents employed (Table 2.2). Where chromatographic resolution permits, the derivatization method employed should minimize the addition of carbon to the analytes, as such additions cause unavoidable increases in the uncertainty of the final isotopic values obtained.

2.4: GC-C-IRMS Overview

Gas chromatography - combustion - isotope ratio mass spectrometry (GC-C-IRMS) is a hybrid analytical technique that uses the resolving power of a gas chromatograph to separate the constituents of complex mixtures prior to the sequential analysis of the component compounds' stable isotopic compositions. Commercial GC-C-IRMS systems have been available for many years. A schematic of such a system is shown in Figure 2.1. Complex mixtures are resolved to individual compounds using an appropriate chromatographic column, which are then sequentially quantitatively combusted to CO_2 and NO_X. Water is removed via countercurrent exchange across a nafion membrane with a stream of dry helium and nitrous oxides are reduced to N_2 gas. CO_2 is optionally removed with a liquid nitrogen trap (for N_2 analysis) and samples are passed into the mass spectrometer across a moveable open split.

Combustion of samples in such a system must occur in an oxygen-rich environment, but surplus free oxygen released into the carrier gas stream can degrade performance of downstream components (specifically the reduction reactor and the IRMS itself). To circumvent this difficulty, oxygen is typically supplied by metal oxides of copper and nickel located within the combustion chamber. At high temperatures (~800 ^{o}C for copper, ~1000 ^{o}C for nickel), oxygen will be donated to combustion reactions directly from these oxides with minimal bleed of oxygen gas into the carrier stream.

Commercially available GC-C reactors are therefore constructed by placing fine copper, nickel, and platinum wires inside an alumina tube of 500 microns inner diameter

(the latter metal serves as a reaction catalyst rather than an oxygen donor). In operation, the reactor tube is mounted inside a resistive furnace and maintained at an operating temperature between 800 -1100^{o}C. Reducing fittings secured with graphite or Vespel ferrules facilitate leak-free attachment to the fused silica capillary lines connecting the GC to the IRMS.

The total mass of metals incorporated into a reactor of this type is quite low. As such, the maximum amount of oxygen that can be supplied from a fully “charged” reactor (i.e., one where all of the surface area of the metal wires within the heated zone has been converted to their corresponding oxides) is also fairly modest. This fact imposes several design constraints on the overall system.

First, there must be a mechanism built into the design by which the reactor can be re-oxidized with an O_2 gas stream while in place, preferably without the need to detach any fittings (since tubing connections are by far the most common source of leaks in GC-C-IRMS systems). During the process, oxygen gas cannot be permitted to propagate downstream (where it will quickly ruin an attached reduction reactor) or upstream (GC columns are highly vulnerable to irreversible oxidative degradation, especially at temperatures above ambient).

Second, the limited amount of oxygen that can be supplied by the combustion reactor must be protected from solvent peaks, which are several orders of magnitude more concentrated in GC injections than are the compounds of interest. A single solvent peak entering a combustion reactor of the type described here can exhaust its entire

oxidative capacity, whereas dozens of samples can typically be run between reactor recharges when solvents are excluded.

Finally, the metal oxides required for combustion are vulnerable to degradation from the formation of metal halide complexes, which reduce the already limited supply of metal available for oxide formation and which are an effectively irreversible reaction in this context (generally referred to as “reactor poisoning”). As such, halogenated analyates are best approached with caution (fluoridated derivatives of organic compounds, despite very favorable gas chromatographic performance, are widely acknowledged to shorten combustion reactor life), and halogenated solvents (i.e., dichloromethane) are best avoided entirely.

To accommodate these requirements, the design of CC-C-IRMS interfaces typically make use of a series of 3- or 4-way low-volume tubing connections attached to pneumatically actuated gas supply lines (Figure 2.1). In “straight” mode, carrier gas flows through the first T-piece (a.) prior to the combustion reactor, then continues through two additional T-pieces (b. and c., respectively) before entering the reduction reactor, water trap, and finally the IRMS open split (Figure 2.2). At each tee, a small quantity of carrier gas is lost to a low-volume bleed flow to prevent the formation of dead volumes (open areas isolated from the main flow into which carrier gas can be entrained) in the attached vent or supply lines. When the system is switched to “backflush” mode, the vent line from the first tee (a.) opens while a helium supply is introduced from the final tee (c.) (Figure 2.2). This reverses the flow path through the

combustion reactor while forcing the column effluent from the GC to vent to atmosphere. In this way, solvent peaks can be vented from the system prior to entry into the combustion chamber, while compounds of interest (which exit the GC substantially later than does the solvent peak) can be retained for analysis via a software-timed event switching between flow modes. Additionally, oxygen gas can be delivered to the combustion reactor for recharging by introduction at a supply tee (b.) with the residual O_2 released to vent away from any other system components (a.).

The GC-C reduction functions to reduce oxides of nitrogen produced in the combustion reactor (NO_X) to N_2 gas prior to entry into the mass spectrometer. Like the combustion reactor, it is built from a 500 micron I.D. alumina tube containing a group of metal wires and operated inside a resistive heater, but the metal used here is reduced metallic copper and the operating temperature is typically 600^{o}C. In contrast to the combustion reaction, here the metal serves as an oxygen acceptor. As such, exposure of the reactor to oxygen gas (either through excessive bleed after combustion reactor reoxidation or from atmospheric leaks anywhere in the GC plumbing) will rapidly destroy it. This must therefore be strenuously avoided, and is the reason for the double-tee piece design downstream of the combustion reactor (b. & c.).

This system has the notable advantage of having no moving parts located within the heated zone of the gas chromatopgraph. GC ovens, due to the rapid temperature cycling occurring during each analytical run, present a very difficult environment for mechanical actuators. Fittings that are gas-tight at low temperatures are prone to seize from

thermal expansion during high-temperature GC runs, while fittings secure at these temperatures are vulnerable to unacceptable leak rates at low temperatures. This difficulty was one of the principal considerations in the establishment of the design described here.

An unfortunate trade-off inherent to this design is the requirement for vent lines to preclude dead volumes at each tee connection. In theory, the volume of analyte lost to these vents should be predictable and trivial (~5% for the total system), but as a practical matter, they have been found (at least with the GC-C-IRMS system located at USF) to cause unacceptable losses of resolution in demanding applications (i.e., N_2 analysis). The system also has an undesirably large number of tubing connections (4 reducing fittings around the combustion and reduction reactors and 3 tee pieces prior to the water trap and open split), any of which present opportunities for leaks to develop. Finally, the alumina reactor tubes used in the two reactors described here are quite vulnerable to breakage during installation, especially given the difficult angles involved with the factory-standard furnace positions. Design changes made to address all of these concerns are outlined below.

2.5: Modifications of GC-C-IRMS

Routine operation of the system described above revealed a series of weaknesses that limited instrument performance and increased the frequency and length of maintenance intervals. These included:

1. short reactor lifespan; limited oxidative/reductive capacity

2. difficulty establishing leak-free flow due to large numbers of fittings connecting fragile components
3. flow management problems relating to the backflush system and elimination of dead volumes

Recent efforts to improve GC-C-IRMS techniques have suggested that the standard combustion-reduction reactor design could be replaced with a single hybrid reactor constructed from metallic nickel of high purity, either with or without copper and/or platinum wires added (Hilkert et al., 2009). At the time this research was done, the manufacturer's intention was to commercialize this design, although the status of that effort is not certain as of this writing.

Retrofitting this improvement into the existing GC-C III design addresses the first two of the shortcomings outlined above. First, the surface area of metallic nickel exposed on the inside of a pure metal tube is many times greater than that available on the surface of the wire bundles used in conventional reactors, thereby increasing the amount of oxygen that can be retained by the reactor in a fully charged state and later donated to combustion. This has the effect of increasing the time period between reoxidation episodes and lessening the impact of solvent peaks and halogenated analyates entering the reactor. Perhaps a greater benefit, however, comes from a surprising bit of fortuitous chemistry: nickel-NiO combustion reactors do not require a separate reduction furnace to resolve nitrogen isotopes, allowing for considerable simplification of instrument design.

Early work in the field of GC-C-IRMS had tested the utility of various reactor designs, and had noted that high-temperature nickel combustion chambers provided reductive capacity for the conversion of NO_X to N_2 absent a dedicated reactor for this purpose (Merritt and Hayes, 1994). The theoretical basis for this capability is thought to be the establishment of an equilibrium between oxidized and reduced metal within the reactor, with the latter serving to operate as a reductive catalyst for the breakdown of oxides. Testing by Thermo Fisher in the course of their design research showed this capability was robust based on sequential additions of N_2O gas to the reactor, with quantitative removal and unbiased isotopic conversion to N_2 observed across multiple samples runs spaced weeks apart (Hilkert et al., 2009).

By virtue of this fact, all use of alumina tube reactors can be eliminated for both carbon and nitrogen GC-C-IRMS. These reactors are fragile, expensive to purchase (~$500 each), difficult to construct if commercial purchases are avoided, and have relatively short lifespans. In contrast, ultra-high purity electroformed nickel tubing of appropriate sizes and tolerances is available inexpensively (Valco Inc.), is sufficiently flexible to aid in making connections to the gas chromatograph and downstream system components, can withstand much higher torque loadings in sealing those connections, and does not experience porosity changes with repeated heating cycles.

A potential problem with this system is the tendency of nickel to form chemical linkages when in contact with alumina at high temperatures (Lourdin et al., 1996). This has the effect of producing a very strong mechanical bond between the nickel tubing

and the support structure within the resistive furnace. For this reason, it is critical to design the heating system in such a way that the tubing support is disposable during reactor replacement. Here, we use a disposable alumina insert containing the nickel tube as well as a removable thermocouple that controls the furnace temperature.

The lifespan of this type of joint combustion-reduction system has yet to be established. Approximately 10-day operational intervals between oxidations have been suggested, although this will depend strongly on the level of usage and concentration of compounds being analyzed, since there is negligible bleed of O_2 from the system when not in use (Hilkert et al., 2009). Reactor replacement should occur after prolonged periods of inactivity or after several weeks of use. The loss of the ability of the system to chromatographically resolve small peaks is an indicator of reactor breakdown.

To address the third shortcoming found in the stock GC-C-IRMS design, technology has been adapted from the field of multidimensional gas chromatography. Multidimensional GC dynamically splits the effluent from a GC column to different outputs, either routing flow directly to a detector or to an additional GC column for further separation. In order to do this, technology has been developed to create high-speed switching values that can withstand the rigors of GC temperature cycling without developing leaks. A pneumatically actuated valve of this type has been installed in the present system between the downstream end of the GC column and the entry into the combustion reactor, where the first tee-piece was formerly located (Figure 2.3). This has the effect of creating a switchable flow path at the end of the GC column, with column

effluent either being carried to the combustion reactor (“straight” mode) or vented to atmosphere (“vent” mode). A supply of pure helium gas continuously purges the alternate flow path, maintaining flow through the remainder of the system when column effluent is vented (Figure 2.3). Systems of this type have been discussed for some time as a means of improving GC-C-IRMS resolution, but have not yet been widely adopted (Brenna et al., 1998; Goodman, 1998).

The hybrid combustion-reduction reactor used for both carbon and nitrogen analysis obviates the need for a dedicated reduction reactor, and therefore allows for the removal of the additional two tee-pieces involved in the backflush and oxygen recharge plumbing. Since oxygen can now traverse the system harmlessly as far as the open split to the mass spectrometer, oxygen recharge can be accomplished by installing a variable bleed of O_2 gas into the purge helium flow upstream of the switching valve and passing the resultant mixture through the combustion chamber with the system in GC-vent mode. When oxygen recharge is not required, the O_2 supply can simply be switched off. During oxidation, the gas stream supplying the reactor is physically separated from the GC effluent, therefore completely removing the possibility of oxygen backing into the GC column and damaging it.

Control of the switching valve is pneumatically actuated. As such, the GC-C-III pneumatic lines intended to control the operation of the backflush and vent valves can be repurposed to operate it, allowing the overall system to operate identically to previous conditions without any need for software changes or scripting.

For measurements of $\delta^{13}C$ using alkane standards at 500 pM concentration, typical precisions obtainable are 0.3 per mil or better (standard deviation). Measurements of $\delta^{15}N$ of individual amino acid standards are precise to 0.8 per mil. Both of these values meet or exceed the listed specifications for the standard GCC design.

2.6: Analytical Procedure

For the inquiries undertaken in this study, samples of proteinaceous material from a variety of sources were first digested to liberate constituent amino acids, purified via column chromatography, derivatized to N-acetyl propyl esters, and finally separated and analyzed using GC-C-IRMS. Each of these steps is detailed below.

2.6.1: Hydrolysis of proteins & purification of amino acids

Complete digestion of proteins is required for amino acid isotopic analysis. This can be accomplished using either acid or alkaline hydrolysis techniques. Acid hydrolysis typically employs immersion in boiling HCl (110^{o} C) under vacuum or inert atmosphere for 24 hours. This method destroys tryptophan and coverts asparagine and glutamine to aspartic and glutamic acids, respectively. Oxidative destruction of serine, threonine, and methionine can reduce yields of these amino acids if hydrolysis is performed under an oxidizing atmosphere. Cysteine is largely converted to cystine by this procedure.

Alkaline hydrolysis employs 2 to 4 M aqueous sodium hydroxide at 100^{o} C for 4 to 8 hours. This technique is less commonly employed, as it destroys cysteine, serine, threonine, and arginine, and causes partial deamination of other amino acids. This is a

critical problem for nitrogen isotopic analysis, as deamination is likely to impart unpredictable fractionation to the recovered amino acids. As such, acid hydrolysis is the preferred method for amino acid isotope studies.

All samples analyzed in this research utilized the acid digestion technique. For each sample, approximately 2-5 mgs of organic matter were immersed in 2 ml of 6 N HCl in a precombusted test tube that was the flushed with N_2 gas and sealed. The samples were heated to 110^{o}C for 24 hours, cooled to room temperature and either evaporated under vacuum (most commonly) or under a stream of dry N_2 gas at 70^{o} C.

Amino acids were separated from overall protein digests through the use of ion-exchange chromatography on Dowex 50WX8 200-400 mesh cation-exchange resin (Metges et al., 1996). Separatory columns were assembled by making a slurry of resin and Milli-Q water, then transferring sufficient quantities of this mixture into precombusted Pasteur pipettes plugged with glass wool to leave 5 cm lengths of resin after settling. At no time were the columns allowed to run dry after assembly. Columns were conditioned with 0.5 N HCl to convert resins to acid form (3x column volume) and then thoroughly rinsed with Milli-Q water (3x column volume). Amino acid samples were dissolved in 2 ml of 0.01 N HCl and 100 µl of 0.01 molar norleucine solution were added as an internal standard. Dissolved samples were pipetted onto chromatography columns and sample vials were rinsed with a further 1 ml of HCl solution which was subsequently added to the column. Under acidic conditions, amino acids were bound

and retained by the resins. Non-amino acid constituents were flushed from the columns with extensive (3x column volume) washing with Milli-Q water.

Amino acids were eluted from the columns with 10 ml of NH_4OH. Under basic conditions, the affinity of the resins for amino acids is broken and they pass out of the column with the solvent. The relatively large quantity of base used ensured full elution of all amino acids, which were collected in precombusted test tubes. A visible pH front was typically observed in the columns during this process, and passed into the collection vessel after 5-7 mls of NH_4OH had been added to the column. The pH of column effluent at this time uniformly reached that of the ammonia solution.

Eluents were evaporated to dryness under vacuum or under an N_2 stream at 70^{o} C. Despite the use of ammonia as an eluting agent, this technique has been shown to have negligible impact on $\delta\ ^{13}C$ and $\delta\ ^{15}N$ values if care is taken to ensure complete elution of all amino acids (Takano et al., 2010).

2.6.2: Derivatization

A two-step derivatization procedure to n-acetyl isopropyl esters was used throughout this study. The chemistry involved closely follows that outlined by Corr et al. (2007). Dry amino acid samples were esterified in sealed test tubes with 2 mls of anhydrous isopropanol acidified with acetyl chloride (4:1 IP-acetyl chloride) at 100^{o} C for 60 minutes. Ester solutions were cooled to room temperature and evaporated to dryness under a gentle stream of N_2 gas. Dried esters were washed with two successive 500 µl

aliquots of dichloromethane (Optima grade) to remove any residual reagents and again dried under N_2.

Amino acid esters were then acylated at all amine, hydroxyl, and thiol groups with a 5:2:1 solution of acetone, triethylamine, and acetic anhydride in sealed test tubes at 60^{o} C for 10 minutes. As before, samples were cooled to room temperature and acylating reagents were removed via evaporation under a N_2 stream, followed by two sequential washes with 500 µl of dichloromethane and subsequent evaporation to dryness.

Derivatized amino acids were purified via liquid-liquid extraction. Samples were redissolved in 1 ml ethyl acetate (Optima) and 1 ml of NaCl-saturated water (Milli-Q) was added to the resulting solution. Amino acid derivatives partitioned into the organic phase, while contaminants and byproducts had greater affinity for the aqueous phase. After vigorous shaking and phase separation, the organic phase was transferred to a clean precombusted 4 ml vial. Extraction of the aqueous phase was repeated with a second 1 ml aliquot of ethyl acetate. The combined organic phase was evaporated to dryness under N_2 at room temperature. The aqueous phase was discarded.

2.6.3: GC parameters

Samples were stored frozen until just prior to injection into the GC-IRMS. Typically, samples were redissolved in 1 ml of ethyl acetate and 100 µl were transferred to a low-volume glass autosampler vial insert. Varying solvent concentration at this step allowed concentration or dilution of samples as necessary for optimal GC-IRMS resolution. For δ ^{13}C measurements, 1 µl splitless injections were used, resulting in a concentration of

analyates on-column that was 0.001 times that in the overall sample. Target concentrations for carbon isotope measurements were 0.2-1 nanomolar, depending on the number of carbon atoms in the molecule.

For $\delta^{15}N$, 5 µl splitless injections were made. Optimization of sample concentrations for compound-specific nitrogen isotopes is considerably more challenging that for ^{13}C, owing to the relatively low concentration of nitrogen in organic compounds (one atom per molecule in most amino acids), the comparative inefficiency of isotope ratio mass spectrometers in ionizing nitrogen, and the diatomic character of the analyate seen by the mass spec (N_2 gas, for which two nitrogen atoms must be combined during sample combustion and reduction). Taken together, these factors increase the required injection concentration of amino acid samples by a factor of 50 to achieve equivalent measurement precision to that of ^{13}C determinations (Hilkert 2003). Gas chromatographic columns are designed to separate relatively low concentrations of analyates and are vulnerable to overloading. This manifests as broadening and distortion of the peaks eluting off the column. In complex mixtures such as amino acid digests of biological samples, this greatly increases the difficulty in obtaining the baseline separation of anaylate peaks required for precise determinations of stable isotope ratios. As such, injection concentrations for $\delta^{15}N$ determination were chosen to strike an optimal balance between peak amplitude (functionally, this is the major determinant of precision for a single compound) and chromatographic separation. Typically, this meant a target concentration of 5-10 nanomoles of each compound on-column.

All samples were analyzed using an Agilent 6890 GC linked to a modified Thermo GCC II-III interface (described previously) and a Finnigan Delta Plus XL isotope ratio mass spectrometer. GC separation of amino acid n-acetyl isopropyl esters is most effective with the use of moderate to high-polarity stationary phases (Meier-Augenstein, 2004; Corr et al., 2007b). Early efforts at $\delta^{13}C$ determinations relied on a RTx -2330 gas chromatographic column (60 meters x 0.32 mm x 0.2 μm) while later $\delta^{13}C$ and all $\delta^{15}N$ analyses were performed using an OV-1701 column (60 meters x 0.25 mm x 0.5 μm). Carrier gas flow was 1.5 mls per minute, constant-flow mode. The GC injector was programmable temperature volatilization design operated exclusively in splitless mode at a temperature of 300^{o}C. The GC temperature program varied with the column used. The Rtx column ramp was 120^{o} C/2 minutes; +4^{o} C /min to 180^{o} C; +2^{o} C/min to 230^{o} C; +20^{o} C to 265^{o} C with a 16 minute hold at the final temperature. The OV column program was 80^{o} C/2 minutes; +40^{o} C/min to 140^{o} C; +3^{o} C/min to 170^{o} C; +6^{o} C/min to 220^{o} C; +40^{o} C/min to 285^{o} C with a 9 minute final hold time. In both cases, the inlet purge valve was opened with a flow of 50 ml/min 4 minutes after injection.

Owing to the loss of the aforementioned amino acids during protein digestion as well as variation in the effectiveness of the derivatizing procedure on different compounds, only a subset of protein amino acids are amenable to analysis using this method. Alanine, aspartic acid (+aspartamine), glutamic acid (+glutamine), glycine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, and valine could all be routinely resolved. Leucine and isoleucine were a potential threat to partially coelute (10-20 seconds peak-to-peak separation) if chromatography was not optimal, as were

proline and serine (20 seconds separation). Other peaks were uniformly baseline resolved with the exception of phenylalanine, which occasionally showed interference in $\delta^{15}N$ due to a small co-eluting contaminant peak. A typical chromatogram for amino acid $\delta^{15}N$ is shown in Figure 2.4.

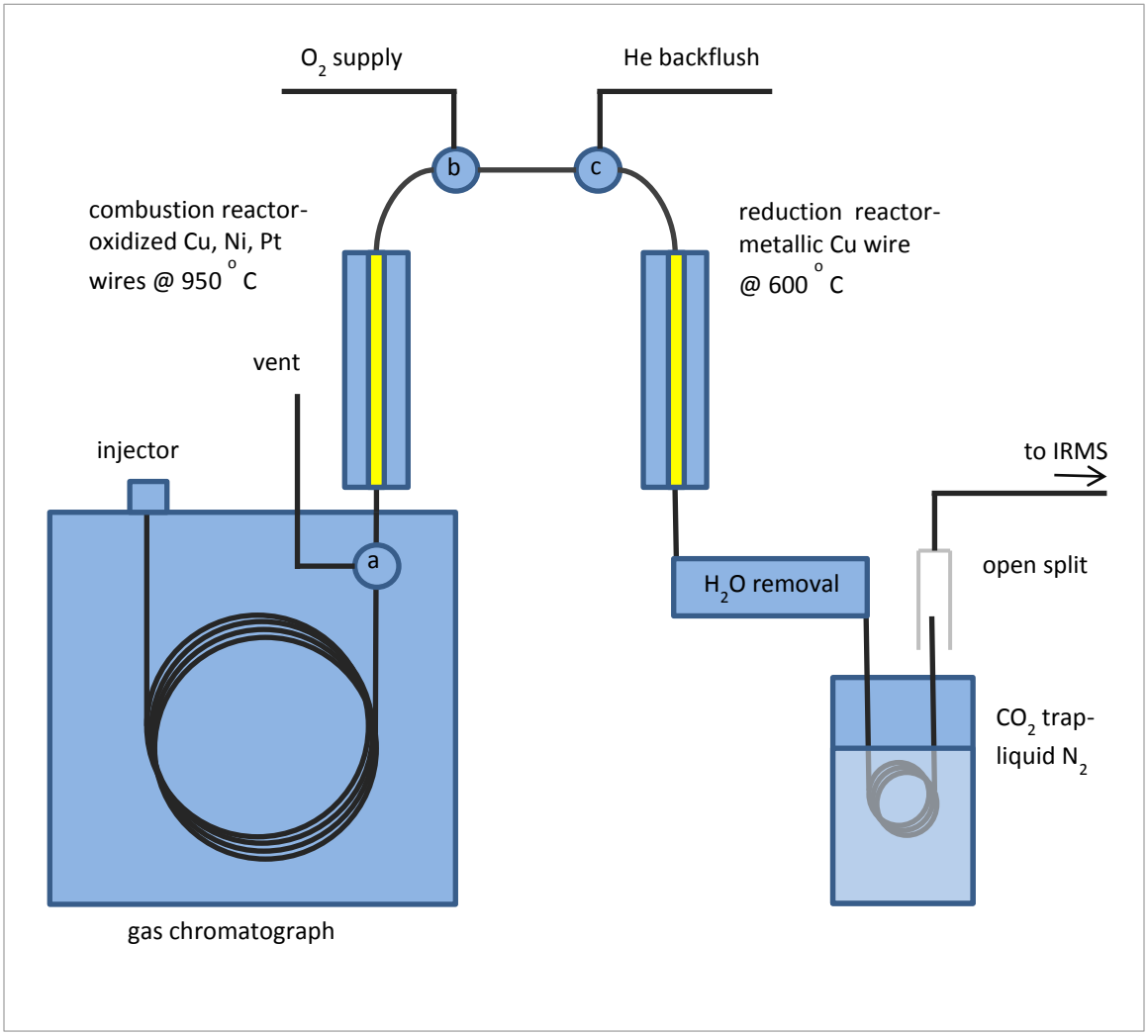
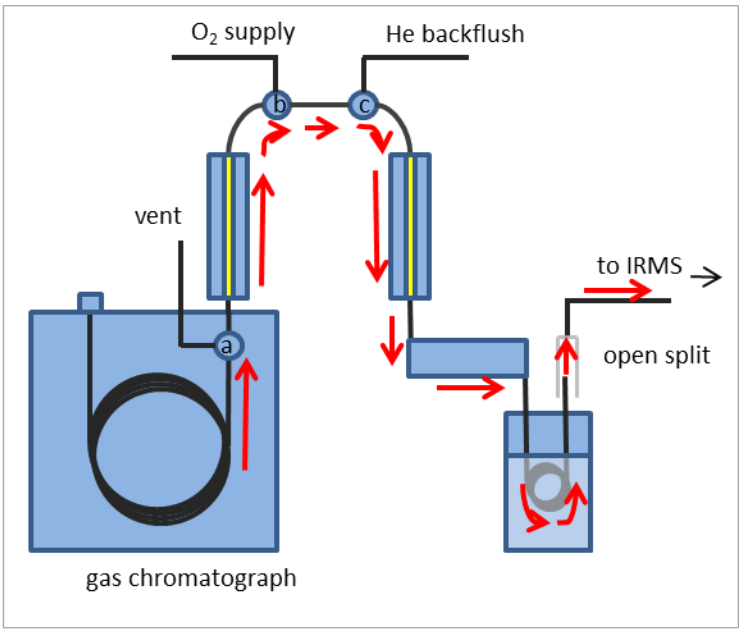
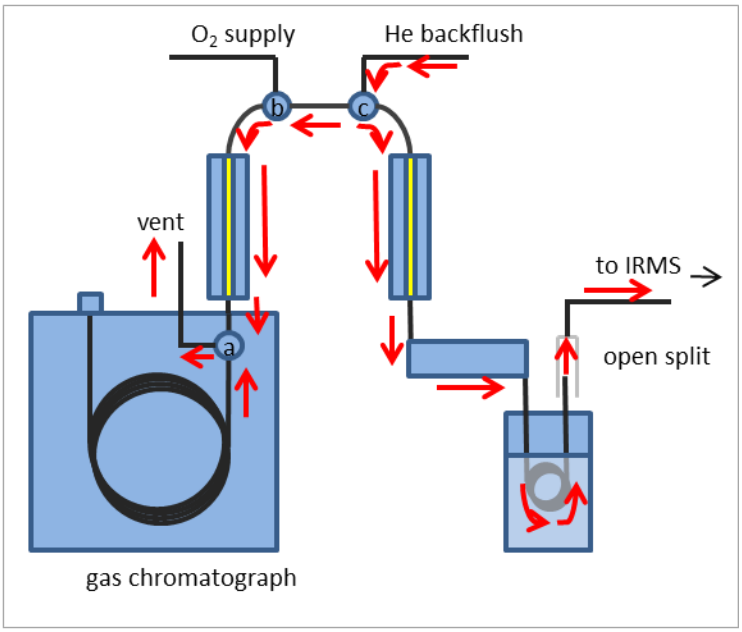


Figure 2.1: GC-C-IRMS schematic. Compounds are separated via conventional GC then quantitatively combusted. Nitrogen oxides are reduced to N2 gas, water is removed, and CO_2 is trapped (if required) prior to admission of samples to the mass spectrometer. Flow path is regulated by gas supplies to a pair of T-piece connections (a. and c.). A third T-piece located between these (b.) allows introduction of O_2 gas to reoxidize the combustion reactor.



2A: GCC straight mode



2B: GCC backflush mode

Figure 2.2: GC-C-IRMS flowpath comparison. In straight mode (A.), column effluent is routed through the combustion and reduction furnaces to the IRMS. A small volume is lost to purge dead volumes at each tee (a., b., and c.). Backflush mode (B.) supplies He at the last tee (c.), blocking flow of column effluent to downstream components and reversing flow through the combustion reactor. Both the backflush and column flows exit via the vent connected to the first tee (a.).

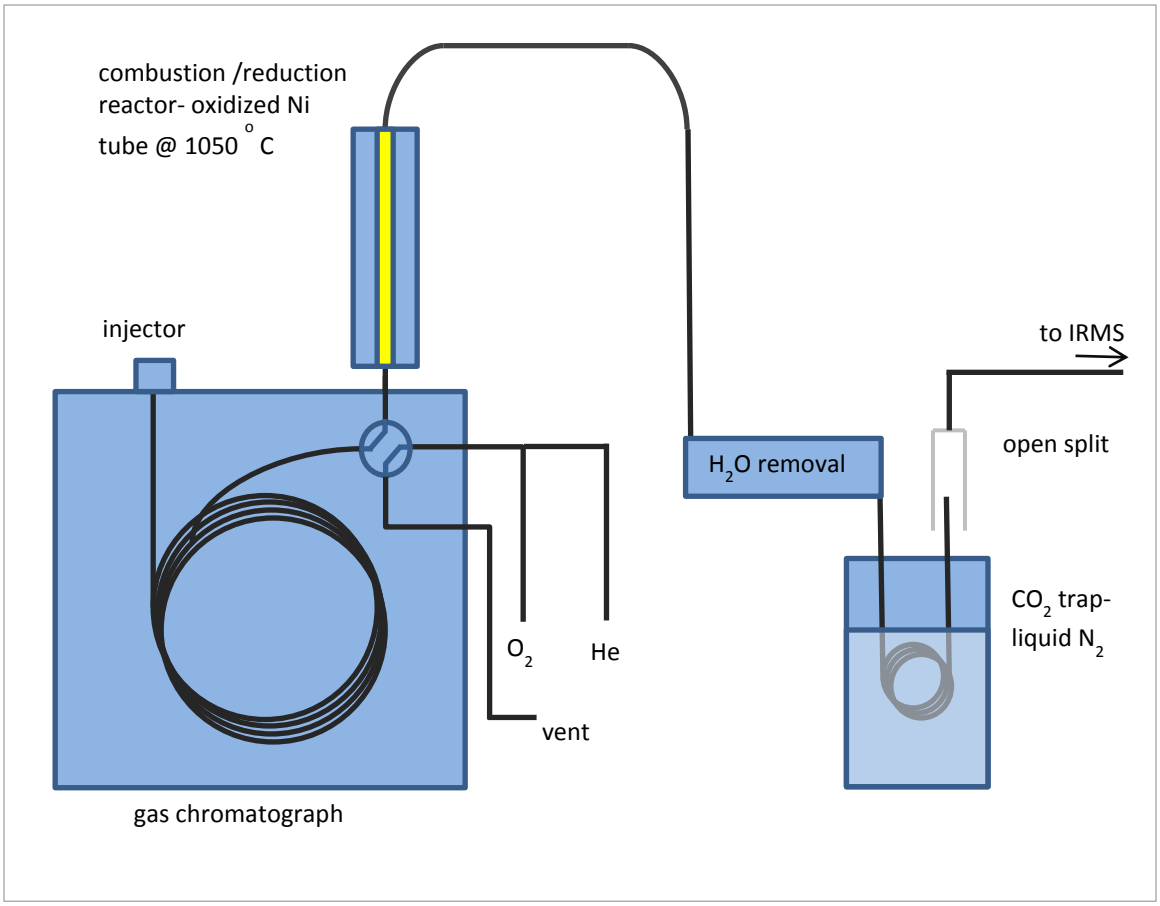


Figure 2.3: USF modifications to conventional GC-C-IRMS design. Backflush system has been replaced with a rotary valve at the terminus of the GC column, allowing selective routing of effluent to the IRMS or to vent. This design is free of dead volumes and associated capillary leaks (all GC effluent reaches open split in straight mode). Replacement of alumina combustion reactor with an oxidized metallic Ni tube eliminates the need for a separate reduction reactor.

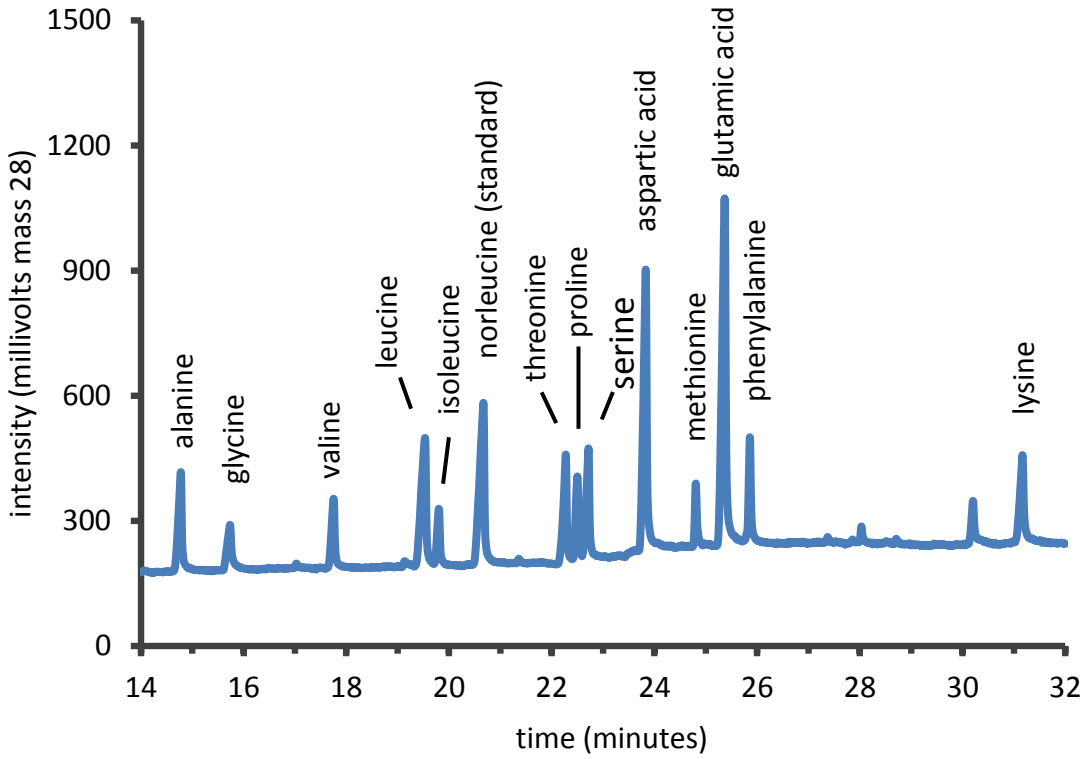


Figure 2.4: Chromatographic trace for a typical amino acid $\delta^{15}N$ determination. Amino acid identities are labeled. Internal standard concentration is 5 nanomoles. Reference gas pulses precede and follow the section shown, but are omitted for clarity.

Table 2.1: Amino acid derivatization requirements. Esterification occurs at all carboxyl groups, while acylation substitutes amine, hydroxyl, and thiol groups. Commonly used derivatization methods are compared. Total carbon additions are lowest for n-acetyl methyl esters (NACME) and highest for n-pivaloyl isopropyl esters (NPIP). N-acetyl isopropyl (NAIP) and trifluoroacetyl isopropyl esters (TFA) are intermediate.

	native	esterification	acylation	carbon additions		
amino acid	carbons	sites	sites	NACME	NAIP/TFA	NPIP
alanine	3	1	1	3	5	8
arginine	6	1	2	5	7	13
aspartic acid	4	2	1	4	8	11
cysteine	3	1	2	5	7	13
glutamic acid	4	2	1	4	8	11
glycine	2	1	1	3	5	8
histidine	6	1	1	3	5	8
isoleucine	6	1	1	3	5	8
leucine	6	1	1	3	5	8
lysine	6	1	2	5	7	13
methionine	5	1	1	3	5	8
phenylalanine	9	1	1	3	5	8
proline	5	1	1	3	5	8
serine	3	1	2	5	7	13
threonine	4	1	2	5	7	13
tryptophan	11	1	2	5	7	13
tyrosine	9	1	2	5	7	13
valine	5	1	1	3	5	8

Table 2.2: Maximum obtainable precisions (‰) for $\delta^{13}C$ measurements of common amino acid derivatives. Calculations assume precisions of 0.1 and 0.3 for EA-IRMS and GC-C-IRMS measurements, respectively.

	maximum precisions		
amino acid	NACME	NAIP/TFA	NPIP
alanine	0.9	1.1	1.6
arginine	0.8	0.9	1.3
aspartic acid	0.9	1.3	1.6
cysteine	1.1	1.4	2.3
glutamic acid	0.9	1.3	1.6
glycine	1.1	1.5	2.1
histidine	0.6	0.8	1.0
isoleucine	0.6	0.8	1.0
leucine	0.6	0.8	1.0
lysine	0.8	0.9	1.3
methionine	0.7	0.9	1.1
phenylalanine	0.6	0.7	0.8
proline	0.7	0.9	1.1
serine	1.1	1.4	2.3
threonine	1.0	1.2	1.8
tryptophan	0.6	0.7	0.9
tyrosine	0.7	0.8	1.0
valine	0.7	0.9	1.1

Chapter 3: Compound-specific isotopic analysis of shell organic matter in *Crassostrea virginica* from Rookery Bay, Florida: A potential archive of environmental data

3.1: Introduction

The analysis of the chemical composition of organic matter in biominerals is increasingly used in paleontological, archaeological, and paleoenvironmental studies. An implicit assumption in any work of this type is that relevant environmental signals captured in the biochemical synthesis of these materials are directly equivalent to those more typically measured in the tissue of the living organisms themselves.

Stable isotope analysis is a tool frequently used to investigate patterns of productivity and nutrient supply based on predictable degrees of isotopic fractionation during metabolic transactions (Peterson and Fry, 1987). The incorporation of inorganic carbon into organic molecules by primary producers results in a characteristic depletion in the heavy isotope, the extent of which is determined by the mechanism of photosynthesis. Carbon isotope ratios carry diagnostic information about the nature of primary producers supporting an ecosystem, and can therefore be used to assess the importance of various classes of producers, such as C_3 versus C_4 terrestrial plants or benthic versus pelagic marine production (Deniro and Epstien, 1978; Rounick and Winterbourn, 1986; Farquhar et al., 1989).

Filter-feeding bivalve mollusks are commonly examined in environmental monitoring efforts due to their sessile nature and continuous 'sampling' of local conditions via feeding (Price and Pearce, 1997; Chase et al., 2001; Fila et al., 2001). Incorporation of dietary signals into the soft tissues of these animals integrates water-column conditions on relatively short timescales governed by the pace of tissue replacement. In contrast, materials sequestered during shell formation provide a continuous record over the life of the organism. Examination of the mineral phase of bivalve shells, along with associated trace element inclusions, has been used in assessing pollution levels as well as in the reconstruction of paleotemperatures (Richardson et al., 2001; Tripati et al., 2001; Surge et al., 2003; Schone et al., 2004; Gillikin et al., 2005). The organic fraction of shells, however, has historically been less commonly utilized as an archival resource.

Calcification in a broad range of eukaryotic organisms, including bivalves, is guided by the formation of an organic template dominated by glycoproteins (Wheeler and Sikes, 1984; Robbins and Brew, 1990; Wheeler, 1992; Nudelman et al., 2005). This organic matrix facilitates mineral precipitation as well as modifying the physical properties of the resulting mineral-organic composite material (Choi and Kim, 2000; Laraia and Heuer, 2005). The proteins involved are tightly incorporated into the mineral structure, leaving them largely isolated from the external environment and slowing the rate of diagenetic alteration (Sykes et al., 1995; Ingalls et al., 2003). The chemical characteristics of this material therefore have the potential to record the signature of the organism's internal biochemistry at the time of shell formation, and are known to be preserved on

timescales far beyond the anthropogenic window (Akiyama and Wyckoff, 1970; Hare et al., 1991; Ostrom et al., 1993; O'Donnell, 2003).

The organic fraction of biominerals therefore offers a useful archive of biochemical and isotopic data across large timescales. Bone collagen from a variety of species has been frequently utilized as a repository of dietary data in archaeological and paleoecological studies (Schoeninger and DeNiro, 1984; Bocherens and Drucker, 2003; Grupe et al., 2009). Organic matrix proteins from modern and fossil *Mercenaria* shells have been used to examine spatial and temporal changes in diet (O'Donnell et al., 2007). Similarly, Mae et al. assessed the degree of chemosynthetic nutritional contributions to fossil bivalve shells from cold seeps via isotopic analysis of shell organics (2007).

A potential problem with this approach is the assumption that biomineral organic residues faithfully record whole-diet carbon isotopic ratios. Controlled feeding experiments using a variety of animal species have shown that the offset between diet and bone collagen bulk $\delta^{13}C$ values are often greater than the typically assumed one per mil, and can vary by as much as six per mil based on manipulations of the proportion and isotopic composition of the protein fraction of the diet (Deniro and Epstein, 1981; Howland et al., 2003). This can be accounted for, at least in part, by variation in the amino acid make-up of the different materials, since different amino acids can vary in $\delta^{13}C$ by 10 per mil or more within an organism (Howland et al., 2003). Since the bulk isotopic composition is by necessity equal to the sum of the weight-proportioned

constituent values, the overall isotopic values of shell organics can be skewed by the dominance of relatively “light” or “heavy” amino acids in the protein structure, without a change in the underlying dietary signal. Analysis at the compound-specific level can therefore give more granularity to the results and assist in comparisons made between tissues.

Compound-specific analysis can also assist with establishing the indigeneity of organic residues in fossil materials. While diagenesis is greatly slowed in mineral-bound organic matter, determining the preservational state of the residual shell organic matrix becomes increasingly important with increasing age (Robbins and Ostrom, 1995). The distribution of isotopic values between amino acid enantiomers has been used as a means to assess contamination and preservational state of organic matter extracted from biominerals (Engel et al. 1994; Silfer et al. 1994). There is evidence of isotopic shifts in amino acid $\delta^{13}C$ values in certain portions of the overall protein component of shell organics during diagenesis (Robbins and Ostrom, 1995; O’Donnell et al., 2007). This may potentially contribute to an alteration of the overall bulk $\delta^{13}C$ of the fossilized organic matter. Focusing analytical effort on amino acids relatively less subject to diagenetic alteration, or on portions of the organic matter pool with the best preservational characteristics therefore can provide a better assessment of the original isotopic composition of the source organism (Sykes et al., 1995).

This study aims to demonstrate that environmentally-relevant signals are recorded and preserved in the carbon isotopic composition of amino acids comprising mollusk

shell organic matrix, and that this information corresponds to that obtained from sampling soft tissues of the living organism taken from natural environments. It utilizes samples of the American Oyster (*Crassostrea virginica*) collected along a salinity gradient in the Henderson Creek estuary of Rookery Bay, FL, over the course of an annual seasonal cycle.

3.2: Methods

3.2.1: Study area & sample selection:

The samples examined in this study were drawn from a series of collections taken in the Henderson Creek estuary at the Rookery Bay National Estuarine Research Reserve during 2007 and 2008. The sampling locations spanned the range of occurrence of *Crassostrea* reefs in the estuary, and were chosen to coincide with the salinity gradient found there. Site 1 was the most upstream location, site 2 represented intermediate conditions, and site 3 was the most seaward reef present (Figure 3.1).

Sampling was conducted on a bimonthly basis. During each field collection, 6 adult *Crassostrea* were randomly collected from each reef, iced, and dissected immediately upon return to the lab. Adductor muscle tissue was removed, dried for 48 hours at 80^{o} C, powdered in an agate mortar and pestle and then stored frozen until analysis. Shells were scrubbed in dilute sodium hypochlorite to remove external contaminants, rinsed with deionized water, and dried for 48 hours at 80^{o} C. The dorsal hinge of each shell was then milled to a fine powder (< 0.5 mm sieve size) with a micromill, and stored frozen.

Bulk isotopic analysis(EA-IRMS) of tissue samples and shell organic matter from samples along this transect undertaken during a previous monitoring study showed persistently depleted $\delta^{13}C$ values at the upstream site, with heavier values occurring at the more seaward sites (Table 3.1). This apparently resulted from contributions of terrestrially derived (C3) carbon to the upper reaches of the estuary overlaid on a background of marine carbon sources at the other locations.

For the present study, a spatially and temporally representative subsample of the total field collection was selected for CSIA. Amino acid extraction and compound-specific carbon isotopic analysis were performed on tissue and shell samples of three *Crassostrea* from each location taken from the August 2007, December 2007, and April 2008 collections.

3.2.2: Amino acid extraction:

Amino acid processing followed the methodology described by Metges et al. (1996). Five mg samples of dried organic material were digested in 4 ml of 6N HCl under a N_2 atmosphere at 110^{o} C for 24 hours. Acid hydrolysis of proteins converts asparagine and glutamine to their equivalent acids (aspartic or glutamic), so these materials are discussed in terms of these acids for the remainder of the study. The digests were then filtered through a 0.7 uM membrane, evaporated to dryness under vacuum, and re-dissolved in 0.05 N HCl. Amino acids were separated from the digests via ion exchange chromatography. The acidified digests were placed at the head of a column consisting of 5 cm of Dowex 50WX8-400 cation-exchange resin in a Pasteur pipette. Under acidic

conditions, this resin binds and retains amino acids. After rinsing with 2 column volumes of 0.05 N HCl to flush non-amino acid constituents in the digests, the amino acids were then eluted with 2 column volumes of 2 M ammonium hydroxide. Samples were again dried under vacuum, rinsed with 500 µL HPLC grade dichloromethane, evaporated under N_2, and kept frozen until derivatization.

Compound-specific isotopic analysis of amino acid samples was conducted via GC-IRMS. Amino acids are not naturally amenable to separation with a gas chromatograph, and therefore must be derivatized to increase their volatility prior to analysis. A number of techniques have been developed in this regard, the most frequently employed of which involve the formation of *N*-pivaloyl isopropyl esters, trifluoroacetyl isopropyl esters, and *N*-acetyl isopropyl esters, respectively. Pivaloylization introduces large quantities of reagent carbon to the anylates, limiting analytical precision, while trifluroaceylated derivatives are thought to degrade GCC combustion reactor performance through the irreversible formation of metal halide complexes (Corr, Berstan, and Evershed 2007). Here, amino acids were converted to n-acetyl propyl esters for analysis (Demmelmair and Schmidt, 1993; Metges and Daenzer, 2000; Corr et al., 2007b). This is a two-step process involving the esterification of the carboxylic acid group and acylation of the amine group of the amino acids, along with any hydroxyl and thiol groups present.

Samples were first esterified in one ml acidified isopropanol (2.8 M with acetyl chloride) under N_2 at 100^{o} C for 60 minutes. The reaction was quenched by placing the

samples in a freezer, and the alcohol was removed via evaporation at room temperature under a gentle stream of N_2. Two 500 µl aliquots of dichloromethane were sequentially added to the resulting amino acid esters and evaporated under an N_2 stream to remove residual reagents and water from the esterification reaction.

The esters were then acylated with 1 ml of a mix of acetic anhydride, triethylamine, and acetone (1:2:5 by volume) under N_2 at 60^{o} C for 10 minutes. After cooling, reagents were evaporated under a N_2 stream at room temperature. Acylated amino acid derivatives were again rinsed with two 500 µl aliquots of dichloromethane to remove any remaining reagents.

Samples were re-dissolved in two mls ethyl acetate to which one ml of saturated sodium chloride-deionized water solution was subsequently added. After vigorous shaking and phase separation, the organic phase of each sample (containing the amino acid derivatives) was transferred to a clean sample vial and evaporated to dryness under an N_2 stream, while the aqueous phase was discarded.

To obtain samples of shell organic matter, 1 g samples of finely powdered whole *Crassostrea* shell were dialyzed (3 KD pore size) against 20 liters of 0.05 N HCl until no visible carbonate remained. Dialysis tubes were then transferred to a 20 liter deionized water bath for 48 hours to remove salts, with complete water changes every 12 hours. The resulting insoluble organic matrix was captured by filtration on a 0.7 micron membrane, dried, weighed, and powdered. Samples were then processed in the manner outlined above for amino acid isolation.

Isotopic analysis was performed using a Finnigan Delta XL isotope ratio mass spectrometer linked to an Agilent 6890 gas chromatograph via a Conflo III interface. Separations were performed using a 60 meter x 320 µM x 0.2 µM RTX 233 chromatographic column. Samples were introduced into the GC via splitless injection at an injector temperature of 250^{o} C. The oven temperature program was 120^{o} C/2 minutes; +4^{o} C /min to 180^{o} C; +2^{o} C/min to 230^{o} C; +20^{o} C to 265^{o} C with a 16 minute hold at the final temperature. Carrier gas was ultrapure helium with a flow rate of 1.5 mls per minute. All values are expressed in standard delta notation relative to VPDB, where:

$$\delta^{13}\mathrm{C} = \left(\frac{\mathrm{R_{sample}} - \mathrm{R_{std}}}{\mathrm{R_{std}}}\right) \mathrm{x}\ 1000\ \ (‰;\ \mathrm{R} = {}^{13}\mathrm{C}/{}^{12}\mathrm{C})$$

Results presented are means of duplicate analyses of all samples. Agreement between replicate analyses was typically better than 0.4 per mil per compound (overall mean =0.38 per mil).

3.2.3: Amino acid $\delta^{13}C$ calculations:

Measured $\delta^{13}C$ values for derivatized amino acids are the product of both the carbon native to the molecule as well as that contributed by the derivatizing reagents. Further, there is a kinetic fractionation during derivatization that causes the resultant isotopic values to diverge from those predicted based on simple mass-balance calculations. The extent of this fractionation varies between compounds, but has been shown to be stable and reproducible regardless of initial isotopic composition and concentration for each individual compound (Silfer et al. 1991). As such, an empirical correction can be

used to circumvent these effects, wherein amino acid standards of known composition (measured via EA-IRMS) are derivatized and analyzed via GC-IRMS, allowing for a calculation of the "effective" isotopic composition of the derivatization carbon:

$$\delta^{13}C_{reagent} = \frac{\left(\delta^{13}C_{A.A.derivative} - (X * \delta^{13}C_{A.A.})\right)}{1 - X}$$

Where $\delta^{13}C_{A.A.derivative}$ is the measured value of the derivatized amino acid, $\delta^{13}C_{A.A}$ is the value of the underivatized standard amino acid, and X is the proportion of the total carbon in the derivative derived from the original molecule. Rearranging terms and plugging in the calculated reagent isotopic values allows for the determination of the amino acid carbon of unknown samples:

$$\delta^{13}C_{A.A.} = \frac{\left(\delta^{13}C_{A.A.derivative} - \left((1 - X) * \delta^{13}C_{reagent.}\right)\right)}{X}$$

A generalized formula for the propagation of error in isotopic measurements of compounds derivatized and corrected in this manner is given by (Docherty et al., 2001):

$$\sigma^2{}_c = \sigma^2{}_S \left(\frac{n_S}{n_C}\right)^2 + \sigma^2{}_{SD} \left(\frac{n_S + n_D}{n_C}\right)^2 + \sigma^2{}_{CD} \left(\frac{n_C + n_D}{n_C}\right)^2$$

where C = the compound of interest

S = the standard used

D = derivatizing agents

SD = derivatized std

CD = derivatized compound

n = the number of carbon atoms in each constituent

In this case, since each unknown compound is corrected using a known standard of the same molecule, this equation simplifies to:

$$\sigma^2{}_c = \sigma^2{}_S + \sigma^2{}_{SD}\left(\frac{n_S + n_D}{n_C}\right)^2 + \sigma^2{}_{CD}\left(\frac{n_C + n_D}{n_C}\right)^2$$

An examination of the structure of this equation demonstrates that as the proportion of analyate to derivative carbon decreases, the total uncertainty sharply increases. Since the molecular structures of amino acids differ in carbon number, and since the number of functional groups requiring derivatization also varies, the absolute limits of uncertainty in the measurements of $\delta^{13}C$ varies between compounds, ranging from 0.7 per mil (phenylalanine, 9 native carbon atoms) to 1.5 per mil (glycine, 2 native carbons).

3.3: Results

A total of 11 amino acids were successfully resolved for all samples analyzed. Table 3.2 shows the average $\delta^{13}C$ profiles for each sampling period and location. Table 3.3 presents analogous data for the shell organic matter samples. Within each grouping, it is readily apparent that differences observed between locations are much more substantial than temporal changes. As was the case with the bulk $\delta^{13}C$ data, there is a trend towards more enriched values moving seaward along the estuary.

To assist with visualizing the relationships in the data, raw $\delta^{13}C$ values were converted to standard scores and used to generate a matrix of Euclidean distances between points for each pair of values. Normalization via standard scores mean-centers each variable and then divides by the standard deviation of that variable, and was done to remove biases in the observed variances introduced by the differing uncertainties of the derivatized compounds. For multivariate data with n dimensions (n discrete values per sample), the distance between any two samples in Euclidean space is:

$$d(a,b) = \sqrt{\sum_{i=1}^{n} (a_i - b_i)^2}$$

Non-metric multidimensional scaling was then used on each dataset to reduce dimensionality and plot the resulting relationships in a two-dimensional plane. The results of this process are shown in figures 3.2 and 3.3 for tissue and shell samples, respectively. In both cases the groupings obtained suggest that position along the transect is the major determinant of similarity between samples, while seasonal effects are much more modest.

Non-parametric, permutation-based MANOVA (MATLAB FATHOM package) of the Euclidean distance matrices was used to test the significance of the observed patterns in the data. Compound-specific isotopic analysis has traditionally been difficult to fit into mathematically rigorous hypothesis-testing schemes due to the presence of large numbers of variables (compounds) in typically modest numbers of samples, making many multivariate solutions impossible to calculate (Boyd et al., 2006). Permutation-

based MANOVA provides a robust solution to these difficulties, with no restrictions on the size of the dataset and no requirement for an assumption of multivariate normality (Anderson, 2001; McArdle and Anderson, 2001). Results of the 2-factor MANOVA (site x time period) tests are given in table 3.4A. In both tissue and shell organic samples, the influence of location on isotopic composition was found to be highly significant (P = 0.0001 for both), while sampling period was not a significant driver of $\delta^{13}C$ (P = 0.083 and 0.090 respectively).

As there were no significant differences detected between periods, sample data were pooled by site and tested using stepwise nonparametric MANOVA to look at relationships between sites. Results are shown in table 3.4B. In both cases, all inter-site relationships showed strongly significant differences ($P < 0.001$). These differences can be clearly seen in plots of the mean $\delta^{13}C$ for each compound at each site. All of the amino acids measured showed a gradient of increasing ^{13}C enrichment moving seaward along the transect for both tissue (Figure 3.4) and shell organic matter (Figure 3.5). Moreover, the extent of this gradient is very similar to that seen in the bulk $\delta^{13}C$ values for both organic pools (the right-most column in each plot).

3.4: Discussion

A clear trend of decreasing $\delta^{13}C$ in all amino acids moving seaward along the transect was observed in both tissue and shell organic matter. The magnitude of this change was approximately equal in both of the organic matter pools sampled. It also paralleled the changes observed in the bulk isotope compositions of each material.

In contrast, no significant effects were seen between time periods. This was true of the compound-specific data in both tissue and shell organic matter, as well as in the bulk isotopic measurements. Shell organic matter represents an integrated sum of dietary inputs across the life of the organism (several years for adult *Crassostrea*), so no effect was expected there, but the invariant nature of tissue values was somewhat surprising given the highly seasonal delivery of freshwater inputs in this region, which receives more than 60% of its total annual precipitation during a four-month summer rainy season lasting from June through September (Florida Climate Center 2011). Internal recycling of terrestrial inputs through the dry season in the upper part of the estuary could potentially account for this, but any explanation for this result based on the available data is speculative in nature.

The patterns observed in the amino acid $\delta^{13}C$ values for both tissue and shell organic matter suggest that the effect of transect position (and, by proxy, terrestrial influence) was uniformly distributed among different amino acids. That is, all of the amino acids measured tended to follow the same patterns of enrichment or depletion with changes in location. This can be shown graphically by plotting the values as residuals after subtracting out the means from each pool (Figure 3.6). The offsets between sites are substantially larger than are those between tissue types. Essentially, the environmental signal was faithfully recorded in both organic reservoirs.

While the overall trends observed in both tissue and shell organic matter amino acid $\delta^{13}C$ were similar, distributions of values were not identical. In particular, aspartic acid,

glutamic acid, and glycine were all relatively ^{13}C-depleted in the shell organic matter compared to the corresponding tissue samples. These three amino acids are typically highly abundant in the organic matrix of calcium carbonate biominerals (Constantz and Weiner, 1988; Ingalls et al., 2003), suggesting that metabolic demand for these materials during shell formation may result in a fractionation relative to other tissues. Alanine was found to have a much more variable isotopic composition in the shell organic fraction than in tissue. The reasons for this are unclear, but as alanine was the first amino acid to elute of the chromatographic column, this may have been a chromatographic effect. Interestingly, the remaining amino acids surveyed were essentially equal in isotopic composition between the two organic matter pools.

It is also noteworthy that the bulk $\delta^{13}C$ values differed substantially between tissue and shell organic matter, with the latter being approximately two per mil more enriched at all locations. This may be due to the influence of non-protein constituents such as polysaccharides involved in shell matrix formation, or to differences in the amino acid compositions of the proteins in each material. The high proportion of acidic amino acids in shell organic matrix, combined with their characteristic enrichment in ^{13}C, suggests the latter explanation is the most likely (Ingalls et al. 2003). The fact that the amino acid values either varied opposite the bulk trend (acidic amino acids and glycine were depleted in the shell material relative to the equivalent tissue compounds, but still enriched compared to the remaining amino acids) or were equivalent suggests that this difference did not originate from differences in the amino acid isotopic values of the two materials.

Direct comparisons between the two organic pools examined here are also complicated by the difference in time periods they represent. The replacement time of protein in soft tissue is on the order of weeks to months, while the organic fraction of shell material is assumed to provide an integrated signal over the entire duration of shell formation. As such, the differences found here may also be influenced by variability in the carbon supply to the organisms over the period prior to this study. Future work could control for this by sampling only shell increments accreted contemporaneously with tissue sampling, but the irregular mode of growth of *Crassostrea* shells makes temporal sub-sectioning of shells difficult.

Few datasets of amino acid carbon isotopes from marine invertebrates exist in the literature with which to compare the present results. Fantle's study of the blue crab, *Callinectes sapidus,* included isotopic compositions of nine of the eleven amino acids measured here, while O'Donnell's work on *Mercenaria* shell organic matter obtained values for eight of them (Fantle et al., 1999; O'Donnell et al., 2007).

The distribution of $\delta^{13}C$ values obtained here is quite similar to that found in *Callinectes*. In body tissue samples, the essential amino acids valine, phenylalanine, and leucine are all sharply depleted relative to bulk values, while isoleucine has an isotopic composition approximately equal to the bulk value. In contrast, the nonessential amino acids alanine, aspartic acid, glutamic acid, glycine, and proline are all enriched relative to bulk values in both studies. In both *Callinectes* and *Crassostrea,* glycine is among the most enriched of all the amino acids measured, with values approximately 10 per mil

heavier than bulk tissue. Serine is also highly enriched in *Crassostrea*, as would be expected based on its close metabolic association with glycine (Lehninger, 1975). Despite the relative enrichment seen in threonine (an essential amino acid not measured in *Callinectes*), the present data suggest a greater degree of biochemical fractionation of non- essential amino acids than essential ones, in agreement with previous studies (Fantle et al., 1999).

Amino acid $\delta^{13}C$ values obtained for *Crassostrea* shell organic matter were similar to those previously found in *Mercenaria* (O'Donnell et al., 2007). Once again, the essential amino acids isoleucine, leucine, and phenylalanine were consistently more ^{13}C-depleted than were the nonessential amino acids measured. Two geographically distinct populations of *Mercenaria* studied by O'Donnell differed substantially in amino acid isotopic compositions (Virginia and Florida), a fact the authors ascribed to phenotypic variability within the species. The values obtained here for Florida *Crassostrea* appear more closely related to those of the Florida *Mercenaria*, suggesting that environmental factors may have played a role in the differences observed between those populations as well.

3.5: Conclusions

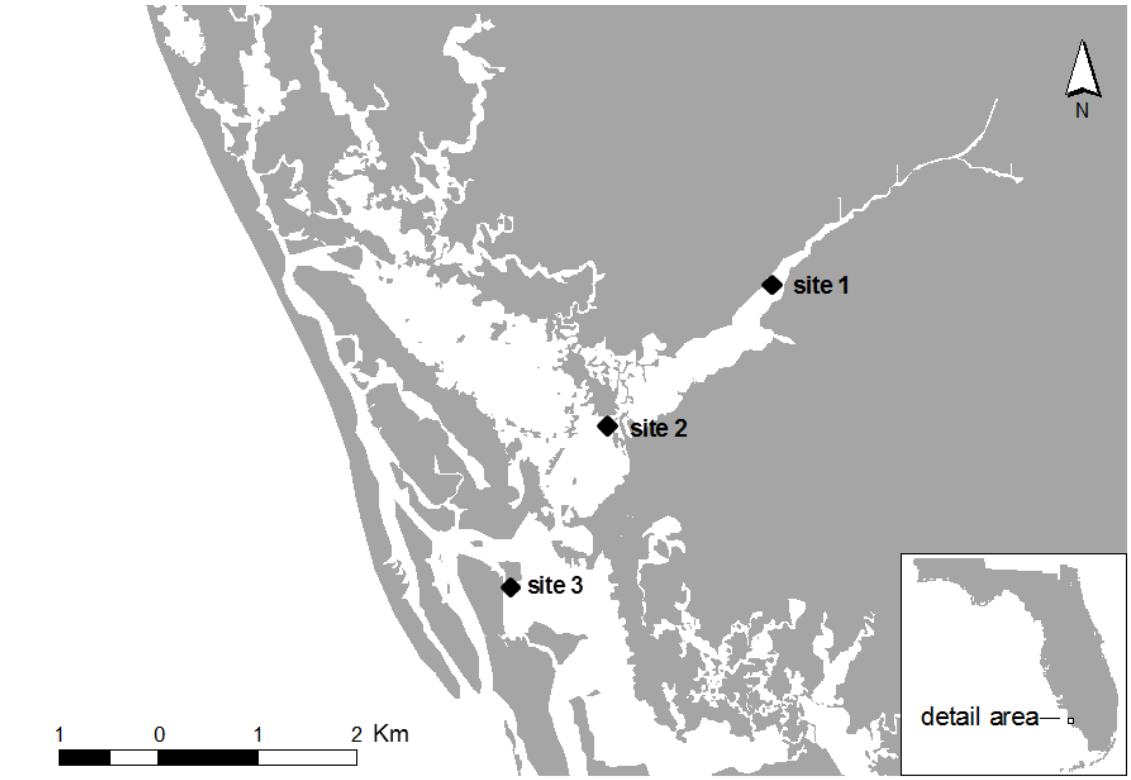
Similar changes in the carbon isotopic composition of *Crassostrea* tissue and shell organic matter occurred across short spatial scales in the area of this study, suggesting that measurements of either material can be useful as a means of assessing carbon sources supporting estuarine or marine production. The differences observed between

sites in bulk isotopic compositions were faithfully reflected in the amino acid constituents of both organic matter pools. The isotopic signatures of the suite of resolvable amino acids were readily distinguishable between sites at high levels of significance through the use of nonparametric, permutation based analysis of variance techniques.

The differences found for the acidic amino acids and glycine between tissue and shell organic matter suggest these compounds may not be ideal for direct interpretation of dietary signals for shell samples for which soft tissue analogues are unavailable (i.e., fossils), and that species-specific patterns of fractionation in different organic matter pools may exist. The remaining amino acids surveyed showed no appreciable offset between organic matter sources and accurately retained the environmental signal. They appear to represent a more straightforward source of information for use in paleodietary studies. The relatively rapid rate of diagenetic loss of acidic amino acids in fossils further reinforces this idea (Bada et al., 1999).

The results of this study show that the organic fraction of biominerals contains isotopic data as suitable for dietary reconstructions as do body tissues. Offsets in bulk isotopic measurements between body tissue and biomineral organic inclusions appear to be absent from much of the available amino acid pool, and these compounds provide a record of environmental conditions in agreement with more conventional techniques. The characteristic pattern of amino acid-specific fractionation relative to bulk isotopic values provides a potential tool for assessing sample integrity and detecting diagenetic

alteration in fossil materials, something that bulk analyses alone are incapable of. While a better understanding of the compound-specific effects of diagenesis is required for accurate interpretation of data from fossil samples and is beyond the scope of this study, the data presented here validates the overall usefulness of this archive.



Map data from Rookery Bay NERR and Florida Geospatial Data Library

Figure 3.1: *Crassostrea* sampling locations, Rookery Bay National Estuarine Research Reserve, FL. Map data from Rookery Bay NERR and Florida Geospatial Data Library.

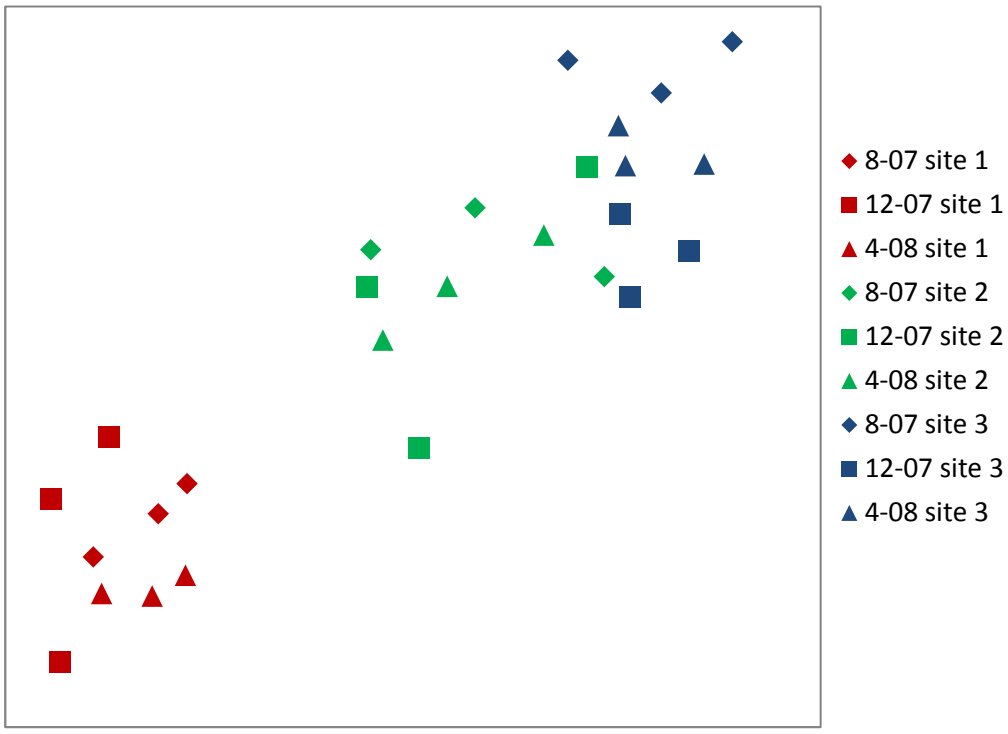


Figure 3.2: 2-D multidimensional scaling plot of differences in tissue samples based on Euclidean distances between standard-score normalized amino acid $\delta^{13}C$ compositions.

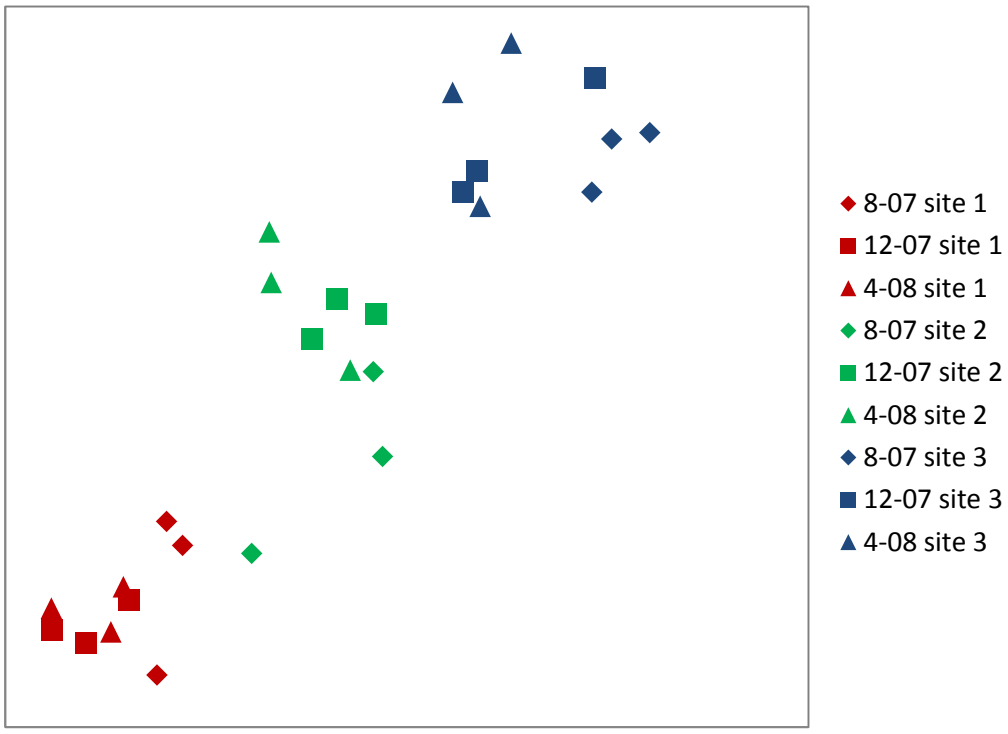


Figure 3.3: 2-D multidimensional scaling plot of differences in shell organic matter samples based on Euclidean distances between standard-score normalized amino acid $\delta^{13}C$ compositions.

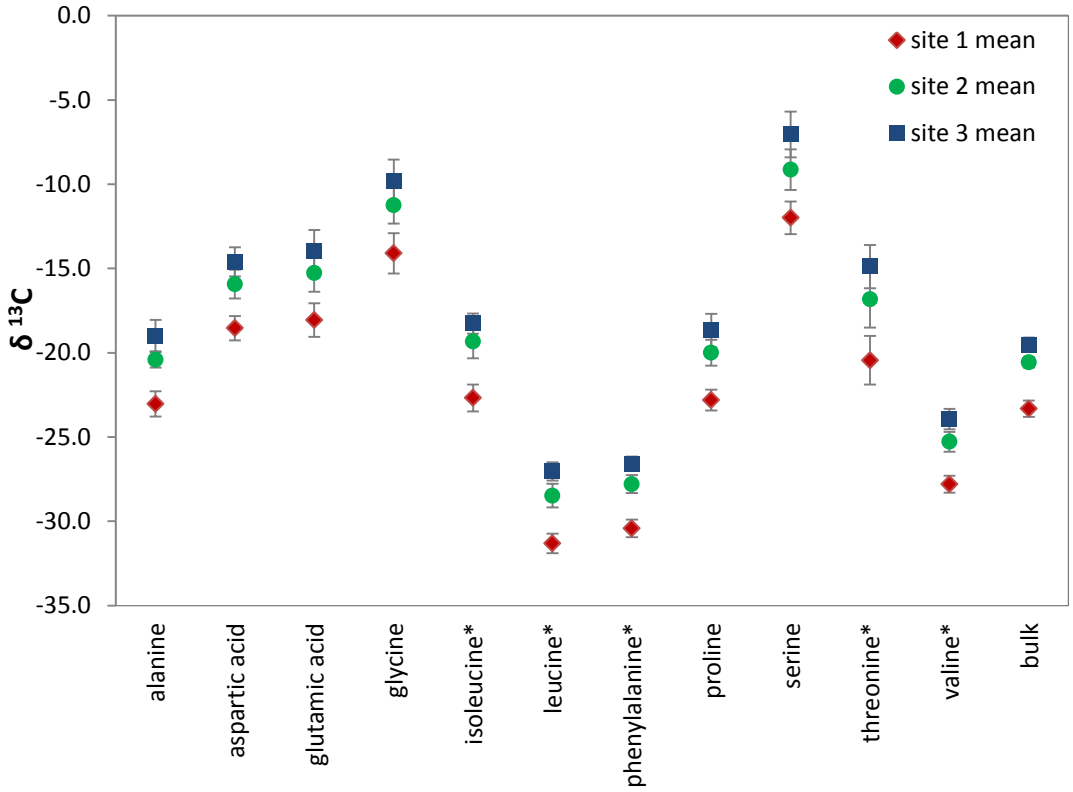


Figure 3.4: Tissue amino acid $\delta^{13}C$ values. Essential amino acids are indicated with an asterisk. Bulk values are from EA measurements. Error bars are standard deviations of all replicates within each site.

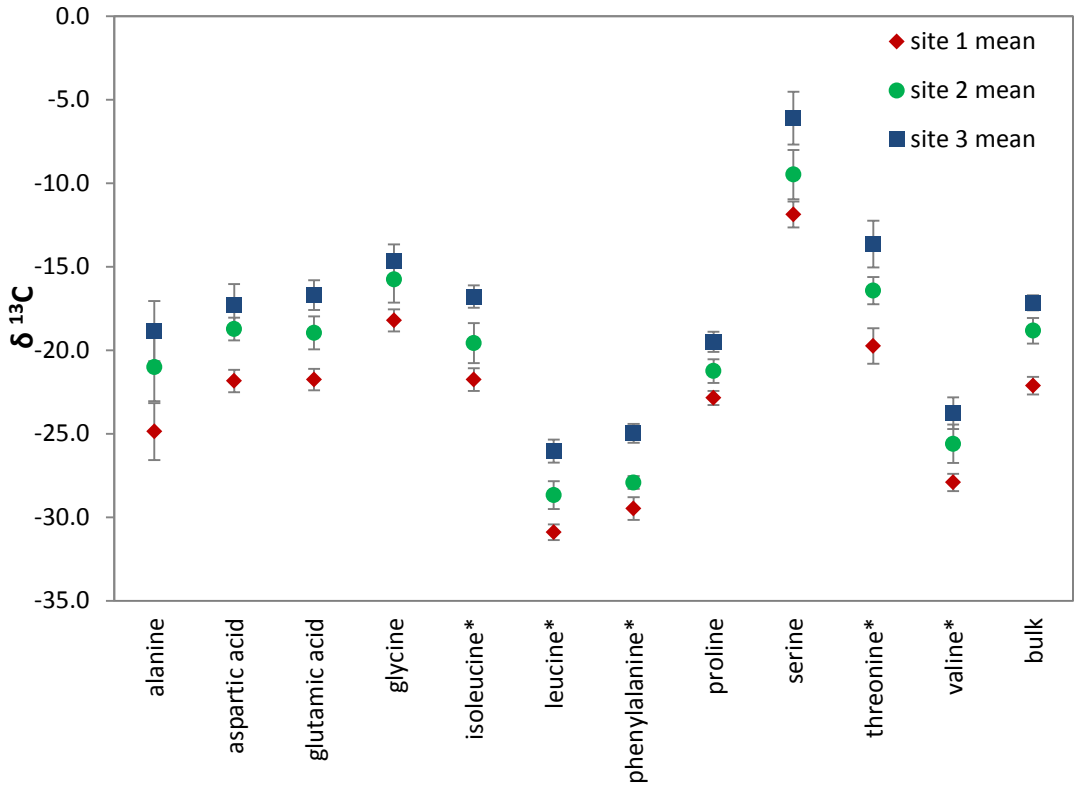


Figure 3.5: Shell organic matter amino acid $\delta^{13}C$ values. Essential amino acids are indicated with an asterisk. Bulk values are from EA measurements. Error bars are standard deviations of all replicates within each site.

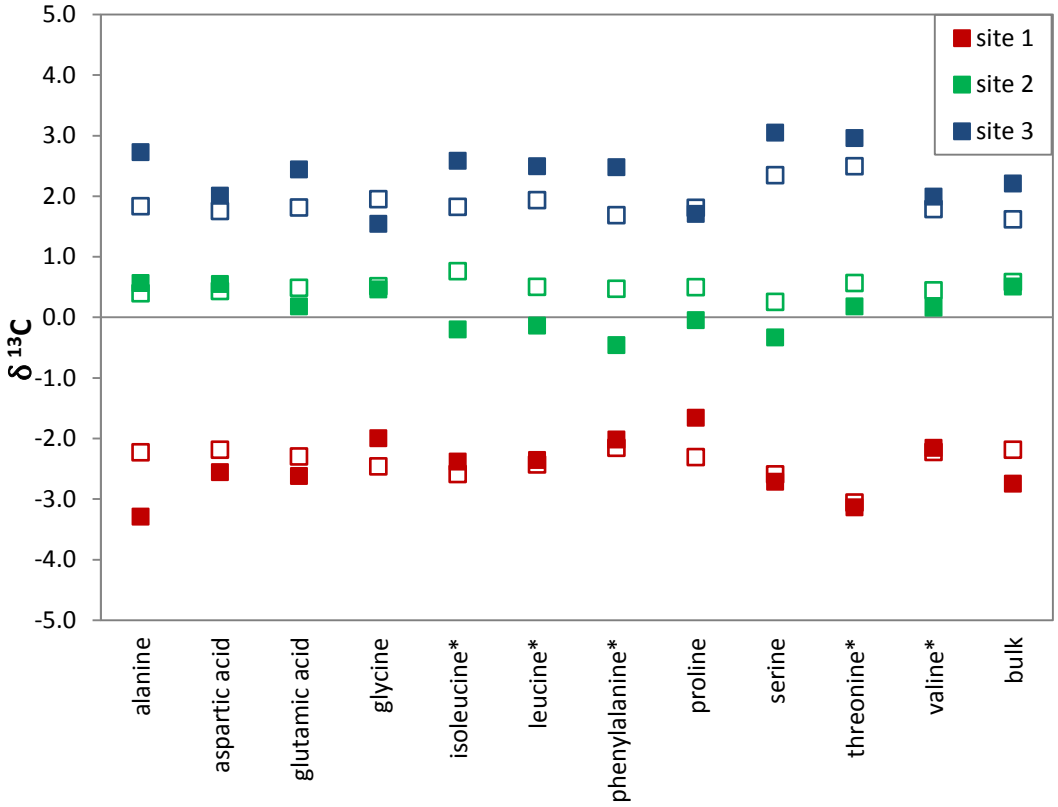


Figure 3.6: Comparison $\delta^{13}C$ values of tissue and shell organic matter amino acids plotted as deviations from within-group means (data are mean-centered). Open symbols represent tissue samples, while closed symbols are shell organic matter. Essential amino acids are marked with an asterisk. Bulk values are from EA measurements.

Table 3.1: Means of bulk $\delta^{13}C$ values for *Crassostrea* tissue and shell organic matter samples, by station and sampling period. Uncertainties are given as standard deviations of the replicates for each sample.

site 1	tissue $\delta^{13}C$	shell organic $\delta^{13}C$
Aug 2007	-23.4 ± 0.5	-21.8 ± 0.5
Dec 2007	-23.4 ± 0.6	-22.0 ± 0.3
Apr 2008	-23.2 ± 0.5	-22.5 ± 0.7
site 2		
Aug 2007	-20.6 ± 0.4	-18.7 ± 0.8
Dec 2007	-20.4 ± 0.4	-18.6 ± 0.5
Apr 2008	-20.7 ± 0.1	-19.2 ± 1.0
site 3		
Aug 2007	-19.6 ± 0.4	-16.9 ± 0.5
Dec 2007	-19.2 ± 0.5	-17.0 ± 0.3
Apr 2008	-19.7 ± 0.3	-17.6 ± 0.2

Table 3.2: *Crassostrea* tissue amino acid $\delta^{13}C$ values, by site and sampling period. Values are average of 3 replicate organisms for each sample. Uncertainties are standard deviations between all replicates for each site. Asterisks indicate amino acids considered essential in animal diets. Aspartic acid and glutamic acid include asparagine and glutamine, respectively, due to conversion during protein hydrolysis.

site 1	alanine	aspartic acid	glutamic acid	glycine	Isoleucine*	leucine*	phenylalanine*	proline	serine	threonine*	valine*
8-07	-22.6	-18.0	-17.8	-13.8	-22.4	-31.4	-30.7	-22.8	-11.9	-19.5	-27.4
12-07	-23.4	-18.5	-17.8	-16.4	-22.7	-31.5	-30.4	-23.0	-12.1	-20.0	-28.1
4-07	-23.2	-19.1	-18.6	-13.3	-23.0	-31.1	-30.2	-22.6	-12.0	-21.8	-27.9
mean	-23.0	-18.5	-18.1	-14.2	-22.7	-31.3	-30.4	-22.8	-12.0	-20.4	-27.8
SD	0.7	0.7	1.0	1.2	0.8	0.6	0.5	0.6	1.0	1.4	0.5
site 2											
8-07	-20.1	-15.3	-15.2	-10.6	-18.8	-28.6	-27.9	-19.7	-9.7	-16.2	-25.1
12-07	-20.5	-16.2	-15.2	-11.7	-19.6	-28.6	-27.9	-20.7	-8.5	-16.6	-25.5
4-07	-20.7	-16.2	-15.4	-11.4	-19.6	-28.3	-27.6	-19.6	-9.2	-17.7	-25.3
mean	-20.4	-15.9	-15.3	-11.2	-19.3	-28.5	-27.8	-20.0	-9.1	-16.8	-25.3
SD	1.5	0.9	1.0	1.4	1.1	0.8	0.7	0.9	0.9	1.0	0.7
site 3											
8-07	-18.8	-14.0	-12.9	-8.5	-17.7	-26.7	-26.5	-17.7	-8.1	-13.6	-23.5
12-07	-19.4	-15.6	-14.9	-10.6	-19.0	-27.6	-26.8	-19.5	-5.7	-16.2	-24.4
4-07	-18.8	-14.1	-14.0	-10.3	-18.2	-26.8	-26.5	-18.9	-7.3	-14.8	-23.9
mean	-19.0	-14.6	-14.0	-9.8	-18.3	-27.1	-26.6	-18.7	-7.1	-14.9	-23.9
SD	0.9	0.9	1.2	1.3	0.6	0.5	0.4	1.0	1.4	1.3	0.6

Table 3.3: *Crassostrea* shell organic matter amino acid $\delta^{13}C$ values, by site and sampling period. Values are average of 3 replicate organisms for each sample. Uncertainties are standard deviations between all replicates for each site. Asterisks indicate amino acids considered essential in animal diets. Aspartic acid and glutamic acid include asparagine and glutamine, respectively, due to conversion during protein hydrolysis.

site 1	alanine	aspartic acid	glutamic acid	glycine	isoleucine*	leucine*	phenylalanine*	proline	serine	threonine*	valine*
8-07	-24.7	-21.3	-21.5	-17.9	-21.1	-30.4	-28.6	-22.7	-11.2	-18.6	-27.9
12-07	-24.9	-22.0	-21.9	-18.8	-22.0	-31.2	-29.9	-23.0	-12.5	-20.4	-27.9
4-07	-25.1	-22.2	-21.9	-17.9	-22.2	-31.1	-30.0	-22.9	-11.9	-20.3	-28.0
mean	-24.9	-21.8	-21.8	-18.2	-21.7	-30.9	-29.5	-22.9	-11.9	-19.7	-27.9
SD	1.7	0.7	0.6	0.7	0.7	0.5	0.7	0.4	0.8	1.1	0.5
site 2											
8-07	-22.6	-19.0	-19.5	-16.3	-20.0	-29.4	-28.0	-21.8	-8.7	-15.9	-26.7
12-07	-21.2	-18.4	-18.5	-14.9	-20.2	-28.5	-27.7	-20.6	-9.2	-17.0	-24.8
4-07	-19.3	-18.7	-18.8	-16.0	-18.6	-28.1	-28.0	-21.3	-10.5	-16.4	-25.3
mean	-21.0	-18.7	-19.0	-15.7	-19.6	-28.7	-27.9	-21.2	-9.5	-16.4	-25.6
SD	2.0	0.7	1.0	1.4	1.2	0.8	0.4	0.7	1.5	0.8	1.1
site 3											
8-07	-17.8	-16.4	-16.4	-14.2	-16.6	-26.1	-24.8	-19.5	-4.3	-11.9	-24.7
12-07	-20.6	-17.4	-16.7	-14.5	-17.0	-26.1	-25.2	-19.2	-7.0	-14.4	-23.4
4-07	-18.2	-18.0	-17.0	-15.3	-16.7	-26.0	-25.0	-19.8	-6.9	-14.6	-23.2
mean	-18.9	-17.3	-16.7	-14.7	-16.8	-26.0	-25.0	-19.5	-6.1	-13.6	-23.8
SD	1.8	1.2	0.9	1.0	0.7	0.7	0.6	0.6	1.6	1.4	0.9

Table 3.4: Results of nonparametric manova tests on mean-centered, variance scaled (standard score) data for tissue and shell organic carbon isotopic compositions.

3.4A. Tests for categorical effects- categories represent spatial (site) and temporal (month) variation.

category	factor	degrees of freedom	F statistic	p
tissue	-site	2	56.6	0.0001
	-sampling month	2	2.69	0.0834
	-interaction	4	0.87	0.51
shell organics	-site	2	67.2	0.0001
	-sampling month	2	2.56	0.0896
	-interaction	4	1.58	0.2

3.4B. Pairwise comparisons between sites, combined across time periods (since period had no significant effect on values, above).

category	site pairing	p
tissue	1 vs 2	0.0001
	1 vs 3	0.0001
	2 vs 3	0.0005
shell organics	1 vs 2	0.0002
	1 vs 3	0.0001
	2 vs 3	0.0001

Chapter 4: Determination of community structure in a multi-species mollusk shell assemblage from Saint Joe Bay, Florida via amino acid-specific nitrogen isotopic analysis of shell organic matter

4.1: Introduction

Stable nitrogen isotope ratios of biological materials are frequently used to address questions of ecosystem structure, trophodynamics, and nutrient cycling (Peterson and Fry 1987; Peterson 1999). Ecosystem baseline nitrogen isotopic compositions reflect various sources and recycling processes of bioavailable nitrogen, while increasing trophic position of consumers imparts a predictable stepwise fractionation on top of such baselines of roughly 3‰ per trophic level (Deniro and Epstein 1981; Minagawa and Wada 1984). Determinations of trophic position using classical nitrogen isotopic measurements therefore are by definition relative measurements. Predators are characteristically enriched in ^{15}N compared to their prey items, but any single measurement without the benefit of context (either from an estimate of ecosystem baseline values or from measurements of a suite of trophically linked organisms) provides limited scope for interpretation.

It is therefore common practice to use low-level consumers to estimate the isotopic values at the base of ecosystem food webs (McKinney et al. 1999; Zanden and Rasmussen 1999; Post 2002). Mollusks are often employed for this purpose, as they are

not mobile over long distances and are typically low trophic-level feeders, characteristics that make them suitable as long-term integrators of ecosystem conditions (Jennings and Warr, 2003; Fukumori et al., 2008). The carbonate shells of mollusks contain an organic fraction which mediates shell formation and physical properties (Choi and Kim 2000). While the body tissues of these organisms are typically employed for the study of present conditions, this organic fraction has drawn considerable interest for examining nutrient dynamics in the pre-anthropogenic period as well as for reconstructing ecosystem dynamics in the geologic past (O'Donnell, 2003; Carmichael et al., 2008). This organic material is well-preserved on century to millennial timescales and has been shown to reflect changes in the environmental conditions encountered by an organism during the period of shell formation (Robbins and Brew, 1990; Sykes et al., 1995; Bada et al., 1999).

A complication that arises with this technique is the complex and variable nature of shell organic matrix itself. As a practical matter, shell organic material is frequently defined as the insoluble organic residue left after acid dissolution of carbonates, often referred to as conchiolin (Bowen and Tang, 1996). Acid-insoluble organics have been shown to be highly correlated with soft –tissue isotopic compositions for samples drawn from the same species, and therefore are useful as proxies for reconstructing environmental changes (McKinney et al., 2001; Mae et al., 2007; Carmichael et al., 2008). While the extraction techniques used to separate conchiolin are simple and straightforward, this sort of analysis does not account for soluble compounds, which often represent a large portion of the total organic matter present in biominerals

(Robbins and Brew, 1990; Robbins et al., 2000; Marxen, 2003). While the amino acid composition of both the soluble and insoluble fractions of shell organic matter are known to vary between species, the former is generally dominated by aspartic acid, glycine, and serine while the latter is often comprised of alanine, glycine, phenylalanine, and tyrosine (Weiner 1979; Sarashina and Endo 1998; Goodfriend 2001; Levi-Kalisman et al. 2001; Miyamoto 2003; Bonucci 2007). Unfortunately, the distinction between soluble and insoluble material is operational in nature, and is sensitive to the specifics of the extraction procedure used (Pereira-Mouries et al., 2002). Comparisons of bulk shell organic matter isotopic compositions between samples of different species are therefore not straightforward, limiting their utility for use in multi-species ecological reconstructions.

While different fractions of shell organic matter vary in amino acid composition, they are all derivatives of the animal's underlying biochemical processes. Individual amino acids incorporated into shell proteins should therefore be isotopically representative of the source organism, regardless of concentration within a particular fraction.

Nitrogen isotopic analysis of protein amino acids provides novel insights into trophic ecology based on differences in patterns of fractionation between compounds (McClelland and Montoya, 2002). The extent of trophic fractionation of amino acid nitrogen is related to the rate at which compounds participate in transamination reactions (Chikaraishi et al. 2009). Amino acids that frequently undergo transamination fractionate sharply up food chains, and have been labeled "trophic" amino acids for this

reason. This category includes major intermediates in biochemical pathways of nitrogen transport and excretion, such as alanine, aspartic acid, and glutamic acid (collectively known as “trophic II” amino acids), as well as leucine, isoleucine, proline, and valine (Hannides et al. 2009). In contrast, amino acids where transaminations are infrequent or absent do not fractionate appreciably during trophic transfers, retaining the isotopic signature of ecosystem nitrogen baseline values. This suite of compounds, known as “source” amino acids, includes glycine, lysine, methionine, phenylalanine, serine, and threonine (Popp et al. 2007). The difference in isotopic composition observed between these two groups within a sample provides and internal measure of absolute trophic position, absent the need for an independent measurement of ecosystem baseline.

Compound-specific nitrogen isotopic analysis of individual amino acids in shell organic matrix proteins therefore provides two potential benefits over corresponding bulk analysis. First, the isotopic composition of each amino acid should reflect that of the pool of that compound available for protein synthesis during shell formation, thereby removing any confounding effect related to changes in relative concentration of each compound in the bulk material. Second, each sample provides a self-contained measurement of the trophic position of the organism in question, providing a means of making interspecies comparisons independent of the absolute values obtained for any single compound.

Here, we compare the results of trophic determinations using both bulk and compound-specific isotopic analysis of soft tissue and insoluble shell organic matter on a

suite of mollusks occupying different trophic positions in the same ecosystem, and evaluate the potential utility of each technique.

4.2: Methods

The samples used in this study were drawn from a collection of bivalve and gastropod mollusks taken in St. Joe Bay, Florida. All animals were collected live and immediately frozen whole prior to analysis. Three predatory gastropods and three filter-feeding bivalves were selected to test the ability to recognize trophic offsets in bulk and compound-specific analysis of both tissue and conchiolin. Gastropod species used were Apple Murex (*Phyllonotus pomum*), Tulip Snail (*Fasciolaria tulipa*), and Lace Murex (*Chicoreus dilectus*), while bivalves were Horse Mussel (*Modiolus modiolus*), Pen Shell (*Atrina rigida)*, and Bay Scallop (*Argopecten irradians*). Two individuals of each selected species were analyzed to test within-species fidelity of isotopic data. A subsample of tissue was removed from the foot of each animal for isotopic characterization, oven-dried at 60^{o} C, powdered and stored in a desiccator cabinet until analysis.

Shell organic matrix samples were obtained by acid dissolution, dialysis, and lyophilization. Shells were first cleaned of adhering material with a dental pick and toothbrush, then soaked in a dilute sodium hypochlorite solution overnight to remove any external contaminants and periostracum. Cleaned shells were transferred to a distilled water bath for 10 minutes and thoroughly rinsed to remove any residual hypochlorite, then over-dried for 24 hours at 60^{o} C.

A small section (approximately 5 grams) was removed from the growing margin of each shell with a Dremel rotatory tool and ground to a fine powder (<0.5 mm sieve size) using an electric micromill. Shells weighing less than five grams were powdered in their entirety. Two-gram samples of powdered shell were then exhaustively dialyzed (3 KD pore size) against 20 liters of 0.05 N HCl for 72 hours while refrigerated at 5^{o} C, with acid bath replacement every 24 hours. Dialysis tubes were then transferred to a refrigerated 20 liter deionized water bath for 48 hours to remove salts, with complete water changes every 12 hours. Organic matter was collected via centrifugation, with the insoluble pellet subsequently lyophilized and stored frozen prior to bulk and amino acid isotopic analysis. All analyses performed utilized insoluble organic matter (i.e., conchiolin) exclusively.

500 µg aliquots of each sample were analyzed for bulk isotopic carbon and nitrogen isotopic composition via EA-IRMS using a CE Instruments NA 2500 elemental analyzer linked to a Delta Plus XL isotope ratio mass spectrometer. For compound-specific stable isotope analysis, 3 milligram samples were hydrolyzed with acid and derivatized to n-acetyl propyl esters. In cases where less the 5 mg of organic matter were available, the entire remaining sample was used.

Samples were digested in 6 N HCl under N_2 atmosphere for 24 hours at 100^{o} C and subsequently dried under vacuum. Acid protein hydrolysis converts the amino acids asparagine and glutamine to aspartic and glutamic acid, respectively, so results represent the combined values of these compounds. Dried amino acids were

redissolved in 0.01 N HCl and 100 µl of 10 µM norleucine solution (aqueous in Mill-Q) were added as an internal standard. They were then separated from residual organic matter by ion-exchange chromatography using Dowex 50WX8-400 resin. Acidified digests were pipetted onto the head of a column consisting of 5 cm of cation-exchange resin in a Pasteur pipette preconditioned with 3 bed volumes of 0.5 N HCl and 3 bed volumes of Mill-Q water. Under acidic conditions, this resin binds and retains amino acids. Columns were flushed with 3 bed volumes of Mill-Q water, then amino acids were eluted with 3 bed volumes of 2 M ammonium hydroxide. Samples were again dried under vacuum, rinsed with 500 uL HPLC grade dichloromethane, evaporated under N_2, and kept frozen until derivatization.

To facilitate gas chromatographic separation, amino acids were derivatized to N-acetyl isopropyl esters prior to analysis (Metges and Daenzer, 2000; Corr et al., 2007b). Amino acids were first esterified with 1 ml of acidified anhydrous isopropanol (2.8M, 4:1 isopropanol:acetyl chloride) under N_2 at 100^{o} C for 60 minutes. Excess reagents were evaporated under a N_2 stream at room temperature, then samples were rinsed with two sequential 0.5 ml aliquots of dichloromethane to eliminate residual traces of reagent.

Amino acid esters were then acylated using 1 ml of an acetic anhydride, triethylamine, and acetone solution (1:2:5 by volume) at 60^{o} C under N_2 for 10 minutes. Residual acylating solution was removed by evaporation with N_2 at room temperature and derivatized amino acids were cleaned with two additional 0.5 ml aliquots of dichloromethane.

Derivatized amino acids were redissolved in 1 ml NaCl-saturated water and 2 ml ethyl acetate. After vigorous mixing, the organic phase containing amino acids was transferred to a clean precombusted 4 ml vial while the aqueous phase was discarded. Samples were evaporated to dryness under an N_2 stream and redissolved in ethyl acetate immediately prior to injection (200 µl for gastropod shell OM, 1 ml for all others).

Samples were injected in splitless mode (4-5 µL injection volume) into an Agilent 6890 gas chromatograph linked to a Finnigan Delta Plus XL isotope ratio mass spectrometer via a GCC-II/III interface. Chromatographic separations were performed using a 60 meter x 0.25mm x 0.5 micron OV-1701 column suited to analysis of n-acetyl isopropyl amino acid esters (Meier-Augenstein, 2004). Injector temperature was 300^{o} C. The GC temperature program operated as follows: 80^{o} C initial, 2 minute hold; 40^{o} per minute to 140^{o}; 2^{o}/minute to 180^{o}; 5 ^{o}/minute to 220^{o}; 15^{o}/minute to 285^{o}, 12 minute final hold. Carrier gas supply was 1.5 mls per minute, constant flow mode. Tissue samples for gastropod molluscs showed a co-eluting contaminant overlapping the peak for phenylalanine using this temperature ramp, so these samples were reanalyzed using a faster ramp (80^{o} C initial, 2 minute hold; 10^{o}/minute to 285^{o}, 10 minute final hold) to obtain corrected values for this compound. Glutamic acid, which eluted adjacent to this peak and which was free of co-eluting compounds, produced identical values (within 0.5‰) using both temperature programs.

Combustion of anaylate peaks occurred in an oxidized pure metallic nickel tube (500 µM inner diameter) containing a copper/platinum wire braid and operated at a temperature of 1050^{o} C. No separate reduction reactor was required (Hilkert et al., 2009). Water and carbon dioxide were removed from the combustion products via a liquid nitrogen trap prior to entry of samples into the mass spectrometer.

System precision and accuracy were monitored via the presence of an internal standard (norleucine) in all samples as well as by the inclusion of a mixed amino acid standard of known isotopic composition at the beginning and end of each run sequence. Measurement precision for the internal standard across the sample series was better than 0.5‰ (overall standard deviation = 0.4‰). Samples were run in duplicate and values presented are means. Agreement between individual sample replicates was generally better than 1‰ (0.6 ‰ for peaks > 250 mV mass 28 intensity).

To quantify species-specific differences in amino acid composition of shell organic matrix, measured mass-28 ion intensities were used to estimate relative abundances of each compound. Since derivatization is known to have variable efficiency for different amino acids, compound-specific response factors were calculated based on ion intensities of standards of known, equal concentration and used to normalize observed sample intensities (Corr et al., 2007b).

4.3: Results

Insoluble organic matter accounted for between 0.1% and 1.8% of shell samples by weight, with bivalves containing substantially higher amounts than gastropods (Table

4.1). This range of organic matter concentration is in keeping with previously reported values (Sykes et al., 1995).

Bulk isotopic analysis of foot tissue samples showed a predictable pattern of isotopic enrichment between bivalves (primary consumers) and gastropods (secondary consumers) in both carbon and nitrogen (Figure 4.1). Observed differences between groups were significant for both isotopes examined (Student's T, $P < 0.01$). In contrast, no discernible pattern of trophic fractionation was present in either carbon or nitrogen analysis of bulk insoluble shell organic matter (Figure 4.2). A full listing of bulk tissue and shell organic matter isotopic compositions is given in Table 4.2.

In tissue samples, nitrogen isotopic compositions were successfully determined for the amino acids alanine, aspartic acid, glutamic acid, glycine, leucine, lysine, phenylalanine, proline, serine, threonine, and valine (Table 4.3). The amino acids isoleucine and methionine were also chromatographically resolvable, but could not be reliably integrated due to low signal intensities and were dropped from isotopic analysis.

Trophic amino acids were significantly enriched in gastropods relative to bivalves ($p < 0.01$, Student's T) by an average of 4.6 ‰ (Figure 4.3). The largest offsets were observed for leucine and proline, with fractionations of 6.3 and 5.8 ‰, respectively. In contrast, no difference was observed between taxonomic groups for the source amino acids glycine, lysine, serine, and phenylalanine (Figure 4.4). Threonine was sharply ^{15}N-depleted in gastropods, suggesting inverse trophic fractionation in this compound. The

amino acid composition of tissue from different species was similar, as estimated from mass 28 integrated peak areas (Table 4.4). Major constituents included aspartic and glutamic acids, leucine, valine, and glycine.

In contrast, the amino acid composition of shell organic matter varied widely between species (Table 4.5). Gastropod shell organics were largely comprised of aspartic and glutamic acids, phenylalanine, and serine. *Modiolus* and *Atrina* both showed a broad suite of amino acid constituents with a large glycine component, while *Argopecten* was predominantly characterized by aspartic acid, with lesser amounts of glutamic acid and serine.

The variable composition of shell organic matter and the small amounts available for analysis limited the number of amino acids for which isotopic compositions could be measured. Chromatographic peaks having mass 28 amplitudes less than 100 mV peak signal intensity were discarded due to poor reproducibility. The remaining values obtained for shell matrix amino acid $\delta^{15}N$ are shown in Table 4.6.

Of the trophic-indicating amino acids, only aspartic and glutamic acids could be reliably determined in gastropod shell organic matter. While somewhat more variable than the corresponding compounds in tissue samples, the mean values obtained for these compounds were strikingly similar (Figure 4.5). Both of these compounds were also resolvable in bivalve samples, along with alanine and leucine. A general similarity was again seen with the corresponding compounds in tissue samples, although aspartic

acid was somewhat more enriched in bivalve shell organic matter, largely due to high $\delta^{15}N$ values in *Argopecten*.

Lysine, serine, and phenylalanine comprised the resolvable source amino acids in gastropod shell organics (Figure 4.6). These were also present in bivalve samples, where glycine was also measurable. While variability between samples was greater, the ranges of values for these compounds were identical to the corresponding tissue samples.

4.4: Discussion

The mismatch in bulk isotopic measurements between tissue and insoluble shell organic matrix shows the difficulties present in using multi-species measurements of this kind. While tissue samples reliably indicated trophic relationships in both $\delta^{15}N$ and $\delta^{13}C$, no such patterns were preserved in shell insoluble organics. Gastropod shell organic matrix was generally ^{15}N-depleted relative to tissue, while bivalves showed either relatively small offsets (*Modiolus* and *Atrina*) or ^{15}N enrichment in shell organic matrix (*Argopecten*).

The species with the best agreement in bulk isotopic values between tissue and shell matrix (*Modiolus* and *Atrina*) had substantially higher organic content by weight than did any of the other species, regardless of taxonomic group (Table 4.1). The shell organic matter in these species also yielded the largest number of resolvable amino acids during compound-specific analysis and showed the smallest compositional differences from corresponding tissue samples (Table 4.5).

Interpretation of the amino acid compositions of various samples requires several caveats. It is critical to understand that these values do not represent the total amino acid content of the samples. They only describe the relative proportions of those compounds that are amenable to analysis using GC-based separations and that can be recovered using acid hydrolysis techniques. As such, arginine, cystine, tyrosine, and tryptophan are all absent, due to the inability of this method to measure them. Asparagine and glutamine are combined into the measured values of aspartic and glutamic acids, respectively.

Arginine, cystine, and tyrosine are all known to be substantial constituents of certain shell proteins, so the proportions obtained here are almost certainly overestimates of the actual totals (Miyashita et al. 2000). They should not be treated as absolute compositions, but rather as relative measures for comparing different samples within the context of this study only. Also, there is an implicit assumption of linearity of detector response with differences in sample concentration. For a variety of reasons relating to the design of isotope-ratio mass spectrometers and gas chromatographic sample interfaces, this assumption is nearly, but not precisely, true. Samples of very low concentration, in particular, may be underestimated.

Insoluble shell organic matrix is generally characterized as being rich in alanine, glycine, phenylalanine, and tyrosine (Bonucci, 2007). The latter of these could not be measured in the present study, but the others, while present, were only infrequently the dominant compounds present in the samples examined here. In contrast, soluble

mollusk shell proteins are noted for having large areas of repeated aspartic acid residues separated by serine or glycine (Weiner and Hood 1975; Weiner 1979). These were large contributors to the total amino acid pool in the shell organic matter analyzed here, regardless of species. These three compounds have been previously found to make up the majority of the acid- soluble matrix in Antarctic scallop species (Halloran and Donachy, 1995). Interestingly, the insoluble matrix of *Argopecten* captured by the extraction technique used in this study had a strikingly similar composition, reinforcing the idea that the partitioning of soluble and insoluble components of shell organic matter is fluid and sensitive to laboratory methods used. It appears likely that a substantial portion of the organic matrix recovered using the present technique was drawn from compounds typically included in the soluble pool.

The patterns of fractionation in nitrogen isotopes of tissue amino acids were in general agreement with established trophic relationships. All trophic-indicating amino acids were enriched in ^{15}N by at least 4 ‰ in gastropods relative to bivalves, while the source-indicating amino acids glycine, lysine, phenylalanine, and serine were indistinguishable between the two groups. Threonine was sharply ^{15}N-depleted in gastropods, suggesting negative trophic fractionation in this compound, a phenomenon seen in other trophic studies as well (Hannides et al. 2009)

The magnitude of fractionation in trophic amino acids was unexpectedly low when compared to previously published compilations of amino acid nitrogen isotopic values in marine organisms (Chikaraishi et al. 2009). The typically quoted trophic fractionation for

glutamic acid (the most commonly measured trophic-indicating amino acid) is 7 ‰, while the baseline isotopic difference in marine autotrophs between this compound and phenylalanine (the canonical source-indicating amino acid) is approximately 4 ‰ (McClelland and Montoya 2002; Chikaraishi et al. 2007; Chikaraishi et al. 2009). These values have been used to define an equation for estimating trophic position as follows (Hannides et al. 2009):

$$TP_{glu\text{-}phe} = [(\delta^{15}N_{glu} - \delta^{15}N_{phe} - 4)/7] + 1$$

When this equation is applied to the current data, the estimates of trophic position obtained were unrealistically low (Figure 4.8). This is partially due to the fact that fractionation of trophic amino acids between primary and secondary consumers was always less than the expected 7 ‰, an outcome made less surprising by the relatively modest (~2 ‰) differences seen in the bulk isotopic compositions. The larger contributors to the low trophic estimates, however, were the isotopic fractionation patterns seen in the primary consumers themselves. Trophic estimates based upon the previous equation for all three bivalve species placed them barely above the expected level of primary producers, suggesting a marked deviation from the generally predicted behavior in this particular system. Whether this is due to the specific species involved or to local environmental factors is unclear, but the latter is perhaps more likely. Herbivorous gastropods feeding on a variety of macroalgal species have previously been shown to fit the above equation well, suggesting that it is applicable to mollusks in at least some cases (Chikaraishi et al. 2007). Environmental influences of compound-

specific trophic estimates are considerably less clear. The datasets used to generate these equations are strongly biased towards pelagic marine autotroph-based systems (Chikaraishi et al. 2009). Behavior in other environments (estuaries, for instance) is less well documented. Recent work comparing organic matter extracted from bones of both terrestrial and marine species has shown that source-trophic partitioning measurements using glutamic acid and phenylalanine do not work for the former group (Styring et al., 2010). Further research is needed into the limits of applicability of current trophic estimation equations.

Shell organic matrix provided a more limited group of resolvable amino acids. Gastropods, regardless of species, had generally similar amino acid compositions, with large contributions from glutamic acid, aspartic acid, serine, and phenylalanine. The bivalves *Atrina* and *Modiolus* had the highest concentrations of shell organic matter, and that material had the widest ranges of amino acids present and was most similar to soft tissues. *Argopecten* samples were predominantly composed of aspartic acid, serine, and (to a somewhat lesser extent) glycine, three amino typically found in soluble shell proteins. The very high proportion of aspartic acid in these samples (a trophic amino acid with typically enriched $\delta^{15}N$ values) may help account for the unexpectedly heavy bulk isotopic values of this species.

Despite the large differences in proportions of amino acids present, the isotopic composition of individual compounds in most cases differed very little between shell organic matrix and soft tissues. Aspartic acid was an exception to this pattern in

Argopecten, where it was enriched by up to 5 ‰ in shell matrix relative to tissue. A similar, but smaller, pattern of fractionation was also seen in *Atrina*. Aspartic acid plays a critical role in biological calcification, and the high metabolic demand for this compound during shell synthesis may account for this behavior (Weiner and Hood 1975). *Argopecten* also showed modestly enriched shell $\delta^{15}N$ in glutamic acid and phenylalanine, a result not seen in any other species (Table 4.6)

Glutamic acid and phenylalanine had similar patterns of fractionation in both tissue and shell organic matrix. The can be seen in a comparison of average $\Delta^{15}N_{glu\text{-}phe}$ values for each species (Figure 4.9). Trophic determinations based on measured differences in isotopic values between these compounds therefore produced roughly equivalent results. This was true even when tissue and shell isotopic values differed (*Argopecten*) and was in direct contrast to the results obtained using bulk isotopic values, where no predictable relation could be observed between tissue and shell organic matrix.

4.5: Conclusions

Shell organic matrix proteins provide a useful archive of biological material from which environmental and ecological information can be recovered through isotopic analysis. Species-specific patterns of isotopic fractionation between body tissues and shell organic matter and compositional differences of shell organic matter can confound interpretation of multi-species data sets, however.

Compound-specific nitrogen isotopic analysis of protein amino acids provides a means to resolve this problem by removing the effect of varying amino acid

concentrations in different organic matter pools. Suites of mollusks occupying two separate trophic levels showed distinct isotopic offsets between source and trophic amino acids in both body tissue and insoluble shell organic matter, despite substantial variability in amino acid concentrations between species. The extent of these offsets differed from that predicted by existing equations for defining trophic position based on amino acid nitrogen isotopic values, suggesting these equations may not have universal applicability in all marine systems. The overall pattern, however, was in clear agreement with existing theory, with substantial trophic fractionation in compounds that actively participate in transamination reactions and little or no fractionation in those that do not.

While further research is needed to determine if the magnitudes of trophic fractionation observed here can be generalized to other mollusk species, it is clear that amino acid nitrogen isotopic analysis of shell organic matrix proteins can provide novel insights into trophic relationships in multi-species assemblages.

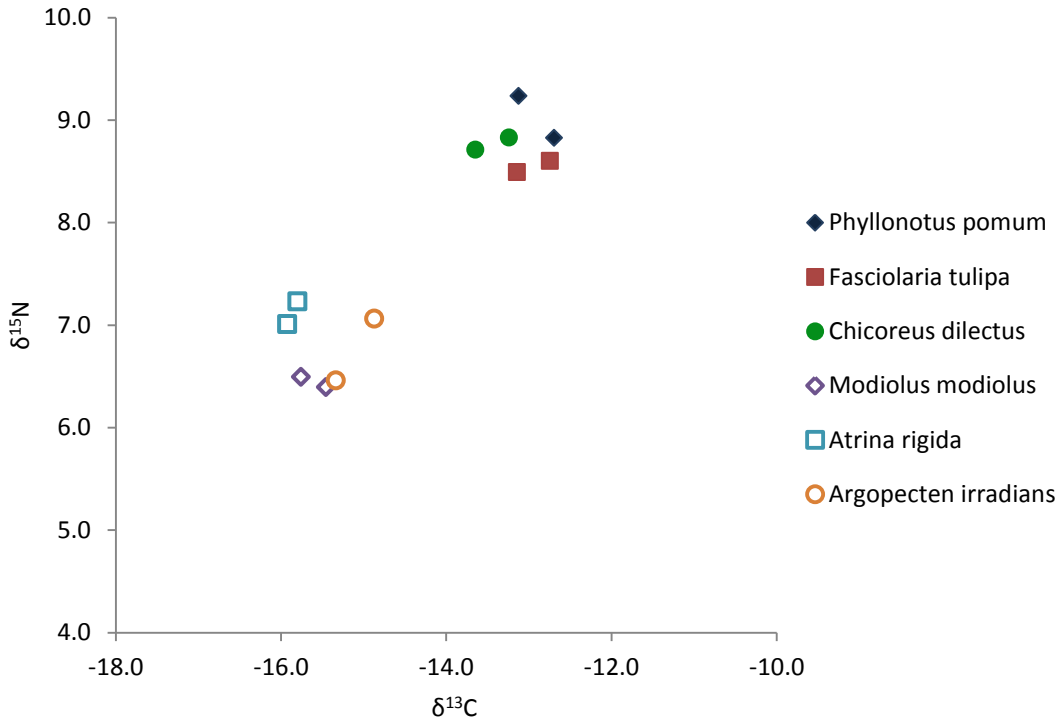


Figure 4.1: Nitrogen and carbon bulk isotopic compositions of mollusk soft tissue samples. Closed symbols designate gastropod species (secondary consumers), open symbols show bivalves (primary consumers).

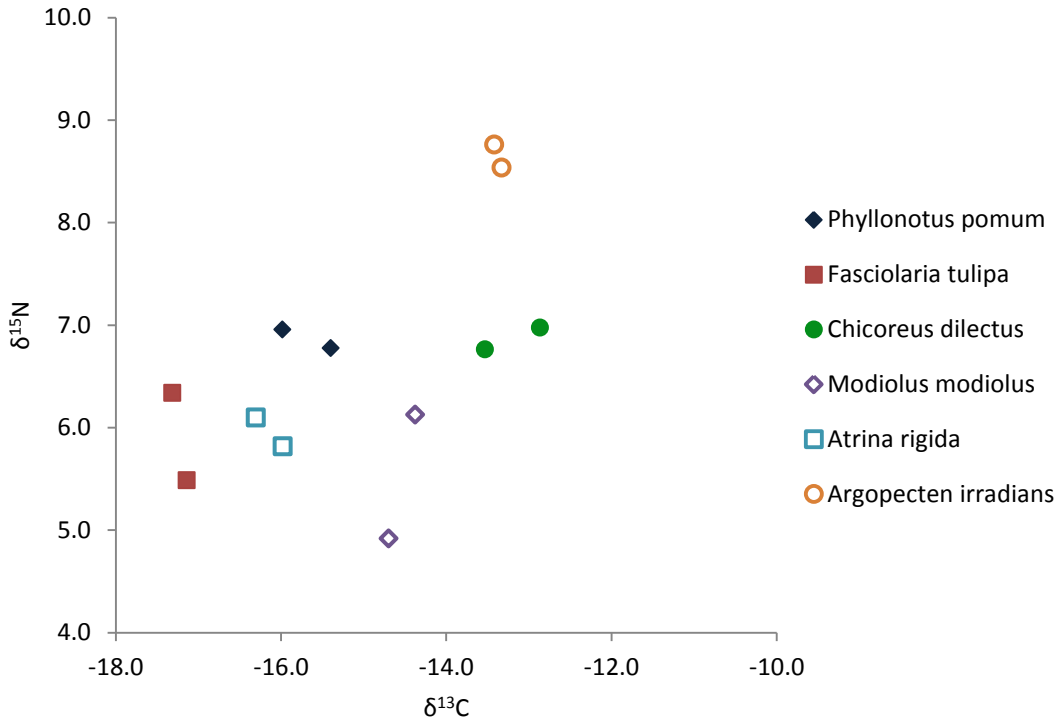


Figure 4.2: Nitrogen and carbon bulk isotopic compositions of mollusk insoluble shell organic matrix samples. Closed symbols designate gastropod species (secondary consumers), open symbols show bivalves (primary consumers).

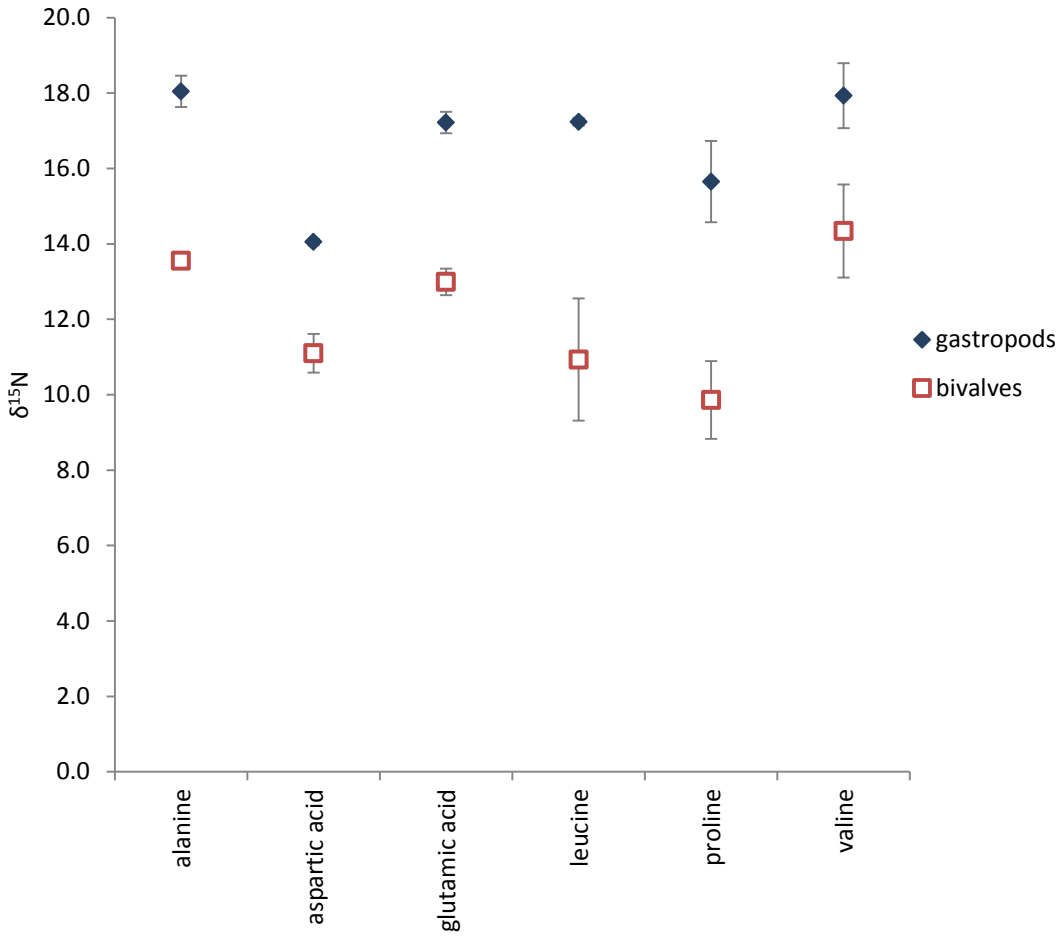


Figure 4.3: Mean nitrogen isotopic compositions of trophic-indicating amino acids in mollusk tissue samples, by taxonomic group. Error bars are standard deviations.

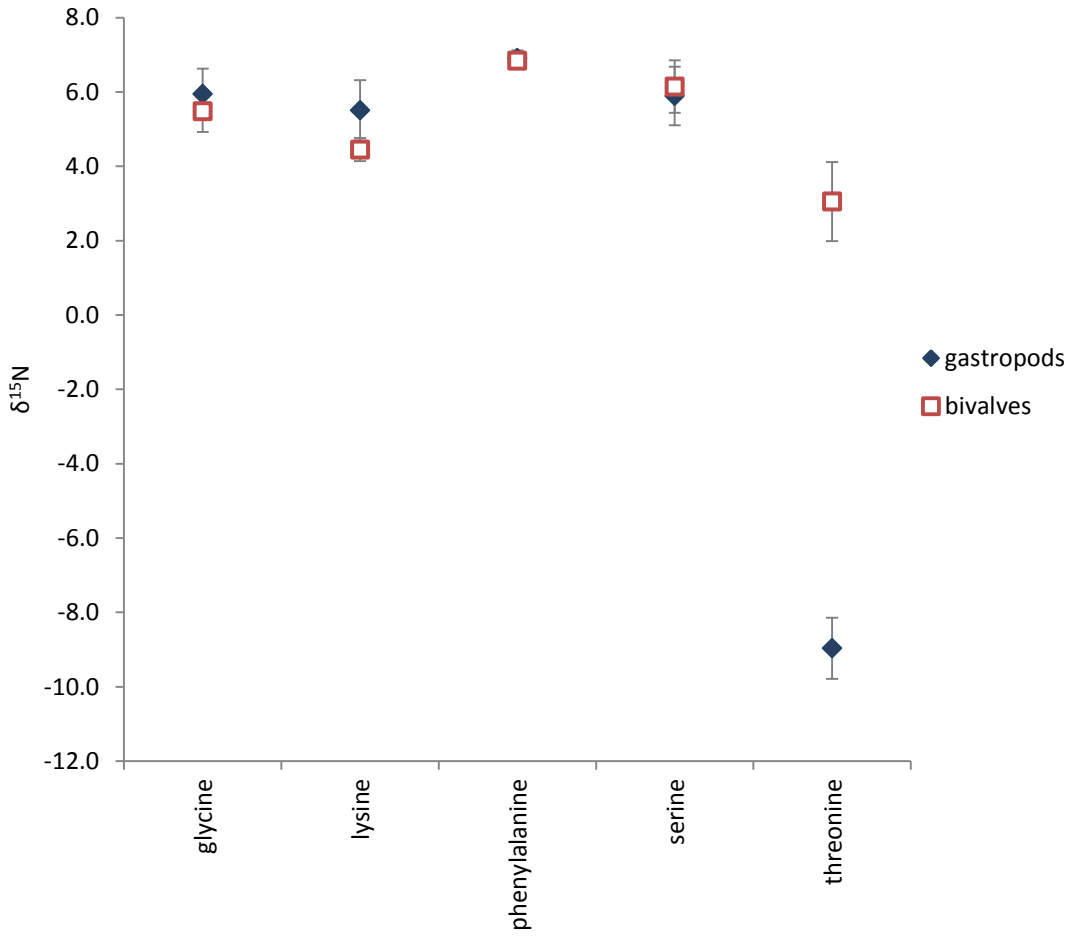


Figure 4.4: Mean nitrogen isotopic compositions of source-indicating amino acids in mollusk tissue samples, by taxonomic group. Error bars are standard deviations.

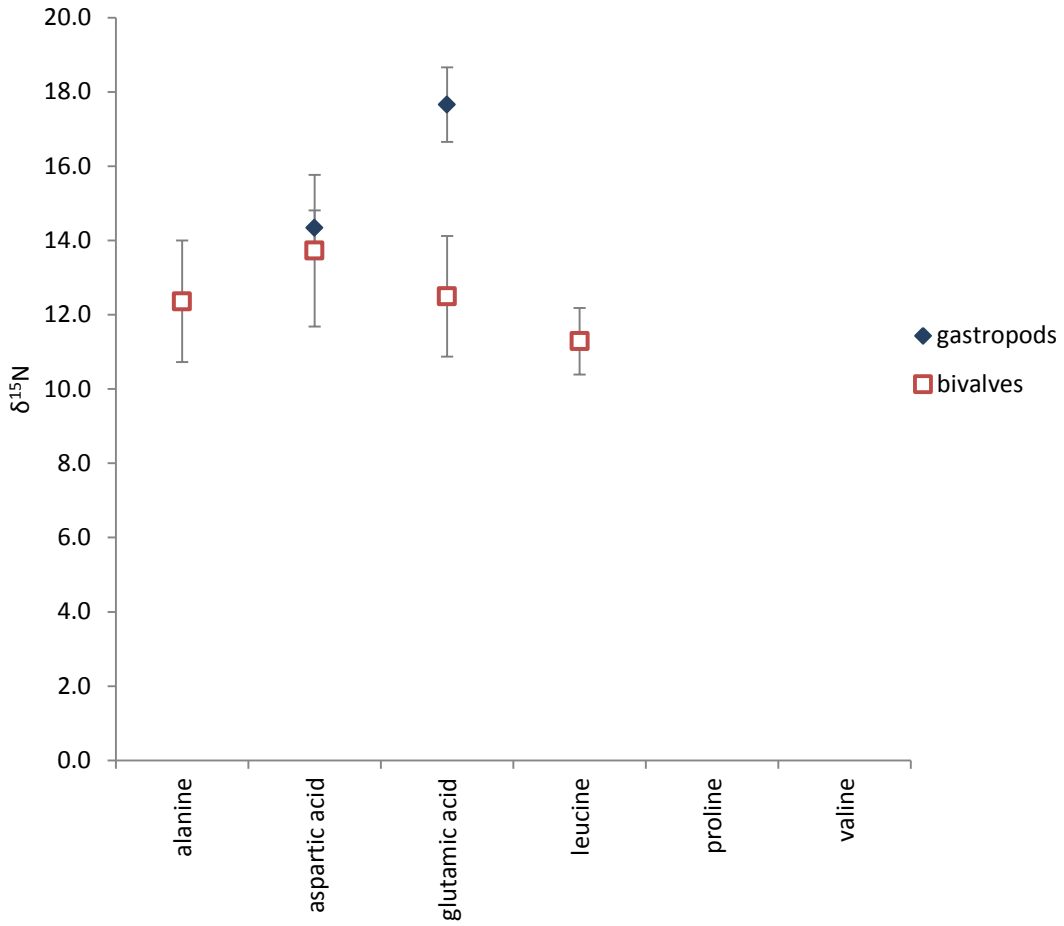


Figure 4.5: Mean nitrogen isotopic compositions of trophic-indicating amino acids in mollusk shell organic matters, by taxonomic group. Error bars are standard deviations. Blanks indicate unresolved or absent compounds.

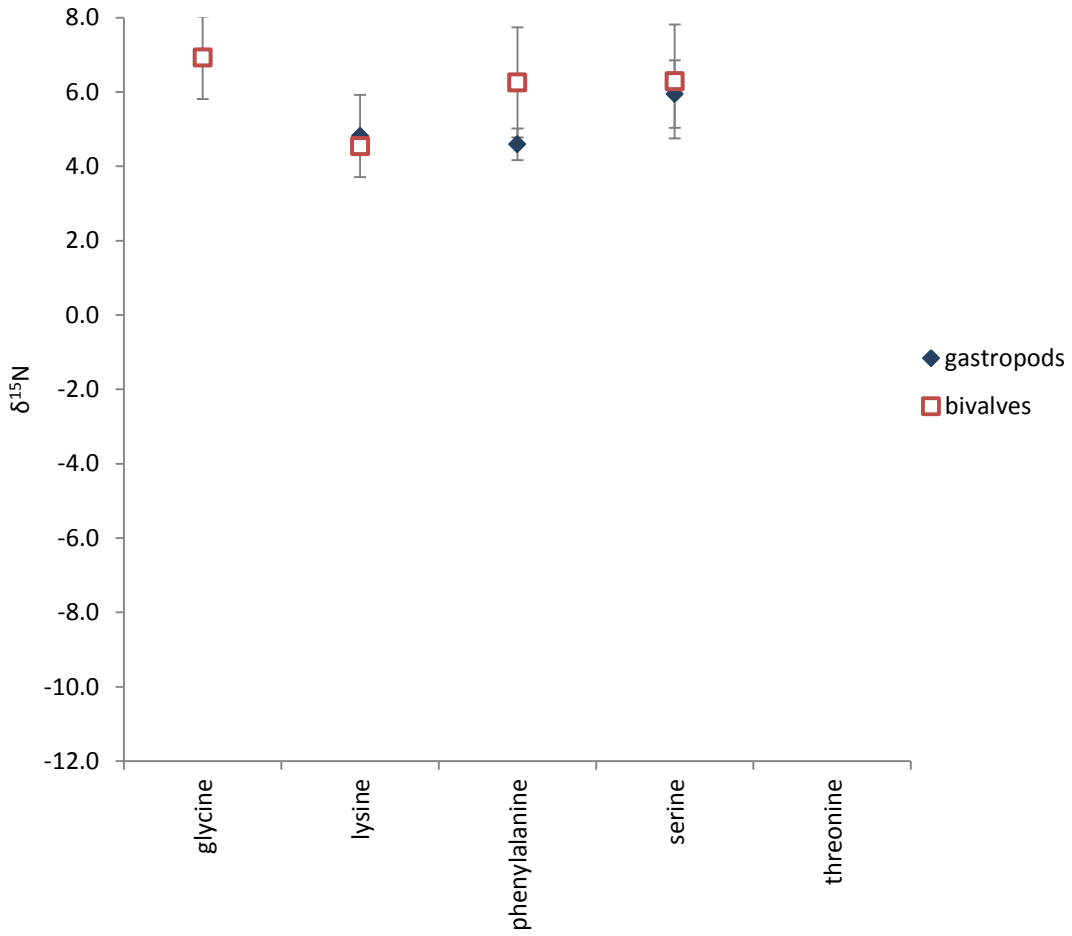


Figure 4.6: Mean nitrogen isotopic compositions of source-indicating amino acids in mollusk shell organic matters, by taxonomic group. Error bars are standard deviations. Blanks indicate unresolved or absent compounds.

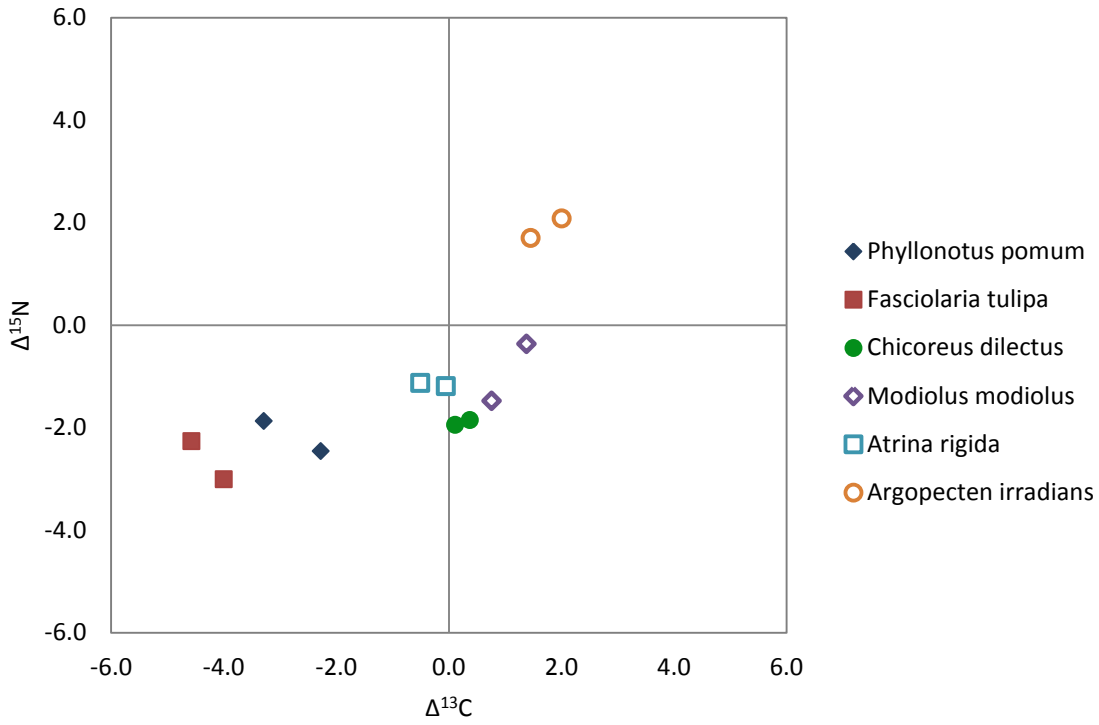


Figure 4.7: Tissue-shell offsets of bulk carbon ($\Delta^{13}C$) and nitrogen ($\Delta^{15}N$) isotopes, by species. Closed symbols are gastropods (secondary consumers). Open symbols are bivalves (primary consumers).

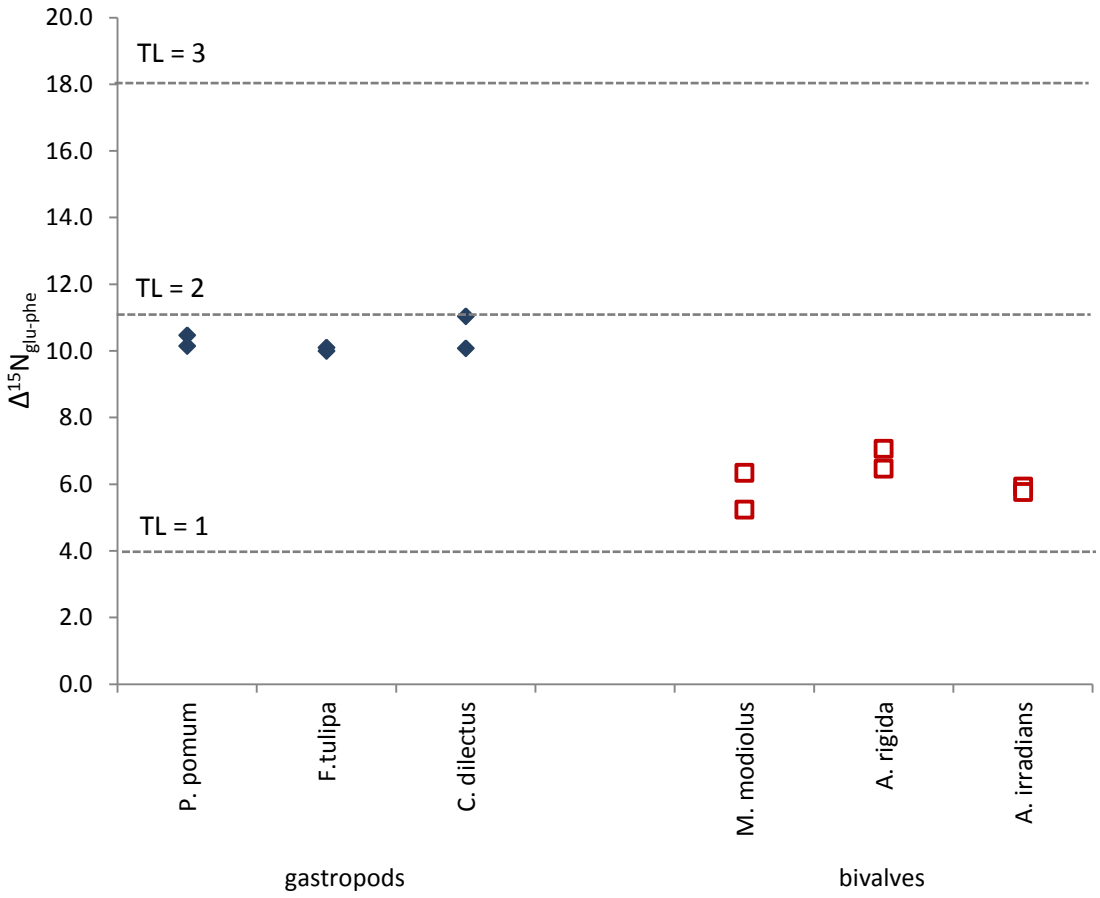


Figure 4.8: $\Delta^{15}N_{glu\text{-}phe}$ values for gastropod (secondary consumers, closed symbols) and bivalve (primary consumers, open symbols) tissue samples. Dotted lines indicate expected values for various trophic levels based on published equation ns (Chikaraishi et al., 2009; Hannides et al., 2009).

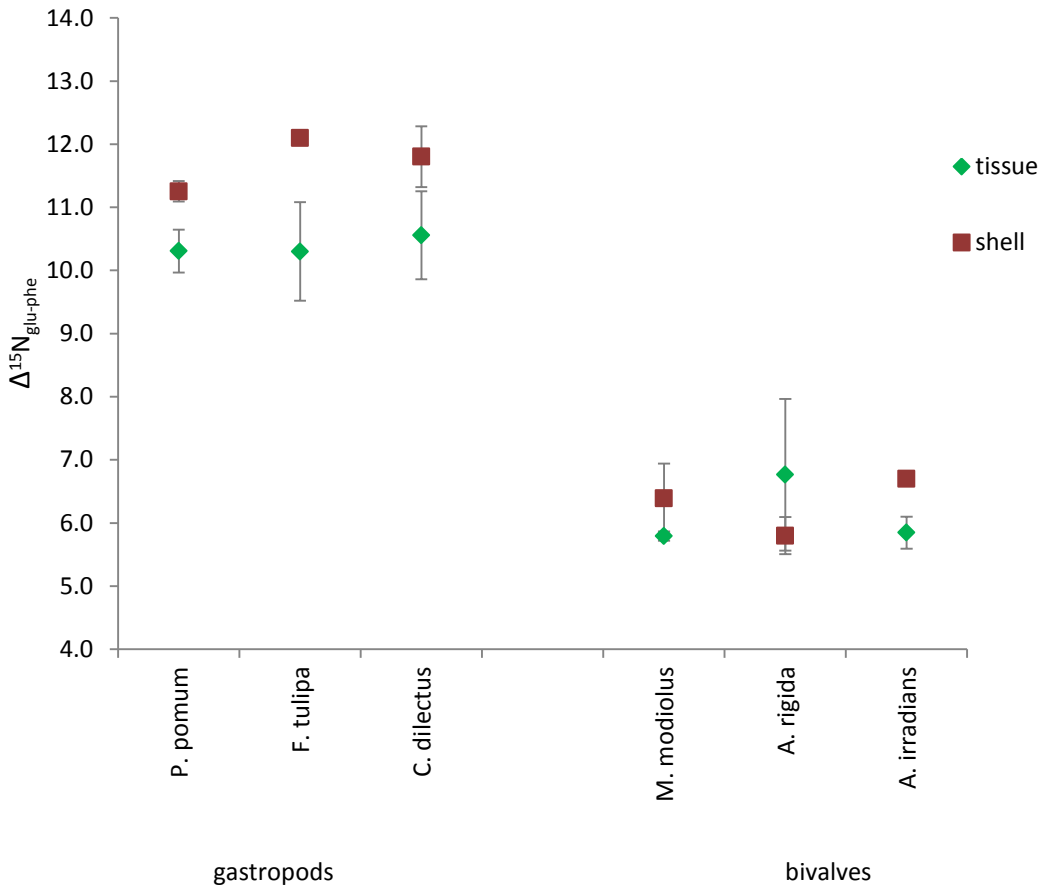


Figure 4.9: Comparison of $\Delta^{15}N_{glu\text{-}phe}$ values for tissue and shell organic matter. Error bars show ranges between replicates for each species.

Table 4.1: Shell masses used for conchiolin extraction and resulting, organic matter quantities obtained. Samples where shell masses are less than 1.95 g are whole-valve weights. SJ1 - SJ7 are gastropods (secondary consumers). SJ39 - SJ69 are bivalves (primary consumers).

ID	species	shell mass (g)	insoluble OM (mg)	weight %
SJ1	*Phyllonotus pomum*	1.98	4.6	0.2
SJ3	*Phyllonotus pomum*	1.97	3.3	0.2
SJ2	*Fasciolaria tulipa*	2.03	5.5	0.3
SJ4	*Fasciolaria tulipa*	1.96	1.7	0.1
SJ6	*Chicoreus dilectus*	2.02	4.8	0.2
SJ7	*Chicoreus dilectus*	2.00	5.9	0.3
SJ39	*Modiolus modiolus*	1.29	17.1	1.3
SJ40	*Modiolus modiolus*	1.01	13.4	1.3
SJ44	*Atrina rigida*	1.97	26.5	1.3
SJ45	*Atrina rigida*	1.99	35.7	1.8
SJ63	*Argopecten irradians*	1.03	4.2	0.4
SJ69	*Argopecten irradians*	2.00	6.8	0.3

Table 4.2: Bulk carbon and nitrogen isotopic compositions of mollusk tissue and shell organic matter samples. SJ1 - SJ7 are gastropods (secondary consumers). SJ39 - SJ69 are bivalves (primary consumers).

ID	species	tissue		shell OM	
		$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$	$\delta^{15}N$
SJ1	*Phyllonotus pomum*	-12.70	8.83	-15.98	6.96
SJ3	*Phyllonotus pomum*	-13.12	9.23	-15.40	6.78
SJ2	*Fasciolaria tulipa*	-12.75	8.60	-17.31	6.34
SJ4	*Fasciolaria tulipa*	-13.15	8.49	-17.14	5.49
SJ6	*Chicoreus dilectus*	-13.65	8.71	-13.53	6.76
SJ7	*Chicoreus dilectus*	-13.24	8.83	-12.86	6.98
SJ39	*Modiolus modiolus*	-15.46	6.40	-14.70	4.92
SJ40	*Modiolus modiolus*	-15.76	6.49	-14.38	6.13
SJ44	*Atrina rigida*	-15.80	7.23	-16.30	6.10
SJ45	*Atrina rigida*	-15.93	7.01	-15.98	5.82
SJ63	*Argopecten irradians*	-14.87	7.06	-13.42	8.76
SJ69	*Argopecten irradians*	-15.34	6.46	-13.33	8.54

Table 4.3: Amino acid $\delta^{15}N$ values for mollusk tissue samples. SJ1 - SJ7 are gastropods (secondary consumers). SJ39 - SJ69 are bivalves (primary consumers).

		trophic AAs							source AAs			
ID	species	alanine	aspartic acid	glutamic acid	leucine	proline	valine	glycine	lysine	phenylalanine	serine	threonine
SJ1	*P. pomum*	16.8	13.5	17.0	17.1	16.5	17.0	5.7	5.2	6.8	6.7	-8.4
SJ3	*P. pomum*	18.8	14.6	17.0	17.3	15.8	17.0	6.0	7.0	6.5	3.4	-8.4
SJ2	*F.tulipa*	18.5	14.5	17.3	17.7	14.0	18.7	5.7	4.5	7.3	5.6	-8.5
SJ4	*F. tulipa*	18.5	13.8	17.0	16.8	14.8	17.6	7.6	4.7	6.9	6.7	-8.7
SJ6	*C. dilectus*	17.5	13.9	17.5	17.7	16.2	18.4	5.1	5.7	6.5	6.4	-10.3
SJ7	*C. dilectus*	18.3	14.0	17.5	16.8	16.1	18.9	5.6	5.9	7.4	6.7	-9.5
SJ39	*M. modiolus*	13.1	10.8	12.5	11.2	9.7	14.9	4.9	3.3	7.2	6.1	0.2
SJ40	*M. modiolus*	14.0	10.2	13.1	10.7	12.2	16.3	7.2	6.3	6.7	5.2	3.6
SJ44	*A. rigida*	13.5	11.4	13.6	12.4	9.7	15.4	6.9	2.6	6.5	6.3	2.7
SJ45	*A. rigida*	13.5	11.3	13.2	12.7	9.7	13.2	4.0	5.8	6.7	7.7	3.7
SJ63	*A. irradians*	14.3	11.0	12.8	9.7	9.8	12.6	4.1	3.9	6.9	6.4	4.1
SJ69	*A. irradians*	12.9	11.9	12.7	8.9	8.0	13.7	5.9	4.9	7.0	5.3	4.0

Table 4.4: Estimated amino acid compositions for mollusk tissue samples from mass 28 peak areas, normalized with response factors from laboratory standards and shown as percentages of total chromatogram integrated areas. Values do not sum to 100% due to small contributions from isoleucine and methionine. SJ1 - SJ7 are gastropods (secondary consumers). SJ39 - SJ69 are bivalves (primary consumers).

		trophic AAs						source AAs				
ID	species	alanine	aspartic acid	glutamic acid	leucine	proline	valine	glycine	lysine	phenylalanine	serine	threonine
SJ1	*P. pomum*	8.0	10.3	19.6	11.0	3.6	9.5	7.9	6.3	3.8	5.7	8.5
SJ3	*P. pomum*	8.1	9.5	19.2	7.9	5.5	10.2	8.2	6.0	4.4	6.3	7.9
SJ2	*F.tulipa*	6.8	8.5	16.1	12.4	2.7	13.4	5.3	8.7	2.8	6.7	9.1
SJ4	*F. tulipa*	6.3	11.2	22.2	9.2	2.9	8.6	5.6	8.5	3.6	6.4	8.8
SJ6	*C. dilectus*	7.4	9.2	17.5	8.8	3.9	10.6	10.0	7.4	4.6	5.5	7.5
SJ7	*C. dilectus*	6.1	10.0	20.0	9.6	3.7	9.9	8.9	6.0	5.6	5.3	7.5
SJ39	*M. modiolus*	5.9	11.2	24.2	10.4	2.5	7.9	7.3	6.5	3.1	6.1	8.0
SJ40	*M. modiolus*	5.7	11.8	23.8	10.0	2.5	8.3	6.7	7.2	3.3	5.4	8.1
SJ44	*A. rigida*	7.5	9.4	18.7	9.2	2.7	10.1	10.2	6.2	3.5	5.7	8.1
SJ45	*A. rigida*	9.5	10.0	19.5	9.6	3.1	9.5	8.8	6.6	3.0	5.9	7.8
SJ63	*A. irradians*	5.9	9.4	17.8	8.5	2.7	8.5	16.3	6.9	3.2	6.1	7.3
SJ69	*A. irradians*	5.6	9.5	18.9	8.1	2.7	8.8	15.7	6.7	3.0	6.5	7.5

Table 4.5: Estimated amino acid compositions for mollusk shell organic matter samples from mass 28 peak areas, normalized with response factors from laboratory standards and shown as percentages of total chromatogram integrated areas. Values do not sum to 100% due to small contributions from isoleucine and methionine. SJ1 - SJ7 are gastropods (secondary consumers). SJ39 - SJ69 are bivalves (primary consumers).

		trophic AAs							source AAs				
ID	species	alanine	aspartic acid	glutamic acid	leucine	proline	valine	glycine	lysine	phenylalanine	serine	threonine
SJ1	*P. pomum*		23.8	35.1					17.7	13.3	10.2	
SJ3	*P. pomum*		22.4	36.3					12.2	11.1	11.9	6.2
SJ2	*F.tulipa*		27.2	46.8						18.8	14.5	
SJ4	*F. tulipa*		27.7	40.6						20.5	13.3	
SJ6	*C. dilectus*		21.5	34.7	4.5				9.6	11.0	9.9	7.4
SJ7	*C. dilectus*		19.8	45.4					7.0	12.4	10.8	4.6
SJ39	*M. modiolus*	5.1	16.7	10.5	6.9	1.8		36.3		5.4	14.0	3.3
SJ40	*M. modiolus*	5.8	20.7	15.0	8.0			23.2		6.3	17.3	3.7
SJ44	*A. rigida*	1.4	18.6	8.5	14.3	1.4		33.7		7.1	9.9	
SJ45	*A. rigida*	1.7	15.4	6.0	12.3	1.7		34.7		6.4	11.0	1.8
SJ63	*A. irradians*		49.4	12.7	1.9			7.7	5.3	2.6	20.4	
SJ69	*A. irradians*		55.1	9.1				7.1		2.3	26.2	

Table 4.6: Amino acid $\delta^{15}N$ values for mollusk shell organic matter samples. Compounds with amplitudes less than 100 mV peak mass 28 intensity omitted. SJ1 - SJ7 are gastropods (secondary consumers). SJ39 - SJ69 are bivalves (primary consumers).

		trophic AAs							source AAs				
ID	species	alanine	aspartic acid	glutamic acid	leucine	proline	valine		glycine	lysine	phenylalanine	serine	threonine
SJ1	*P. pomum*		14.3	16.9							5.3		
SJ3	*P. pomum*		15.2	17.9						3.5	7.0	4.3	
SJ2	*F.tulipa*		13.8	18.9							7.2		
SJ4	*F. tulipa*		14.2	18.1							5.7		
SJ6	*C. dilectus*		14.2	16.1						5.6	5.0		
SJ7	*C. dilectus*		14.4	18.0						5.1	5.5	4.9	
SJ39	*M. modiolus*	13.0	11.2	11.1	12.5				7.0		4.8	4.7	
SJ40	*M. modiolus*		11.7	11.5	10.4				8.6		5.1	5.8	
SJ44	*A. rigida*	10.5	14.1	12.6	11.3				5.8		5.5	4.5	
SJ45	*A. rigida*	13.6	13.6	11.0	10.9				6.1		6.5	7.4	
SJ63	*A. irradians*		16.6	14.8					7.1	4.6	8.8	7.9	
SJ69	*A. irradians*		15.1	13.9							7.0	7.3	

Chapter 5: Detection of ontogenetic diet shift using amino acid nitrogen isotopes: A case study of silver perch, *Bairdiella chrysoura*.

5.1: Introduction

Diet shift during early fish growth has been documented for a wide variety of both fresh and saltwater species (Winemiller 1989; Bergman and Greenberg 1994; Labropoulou 1997; Garcia-Berthou and Moreno-Amich 2000; Graham et al. 2006). These ontogenetic diet transitions, which can be highly consistent and therefore predictable, allow rapidly growing juvenile fishes to avoid strongly suboptimal diets and seasonal prey shortages, thereby markedly improving survival (Post, 2003). Such shifts are usually discovered by comparing stomach-content data across a series of length classes, an approach that yields information-rich results at the expense of large labor investments and biases associated with short-term feeding trends and stomach retention times that vary with prey size and type (Scholz et al., 1991; Renones et al., 2002).

Stable isotope analysis provides a powerful complementary technique to address these shortcomings, as it integrates trophic position over longer periods and is based on the well-established trend of enrichment in ^{15}N with increasing trophic position (Deniro and Epstein, 1981; Minagawa and Wada, 1984). For multiple samples drawn from the same food web, trophic enrichment of ^{15}N makes establishing relative trophic position fairly straightforward, at least to the limits that trophic baselines and fractionations are

known (Vander Zanden and Rasmussen, 2001; McCutchan et al., 2003; Bloomfield et al., 2011). Accurate determinations of trophic position require estimates of central isotopic tendencies at or near the base of the food web from which fish samples are collected (i.e., at or near basal resources,Post 2002). The most common method for establishing the isotopic baseline of basal resources is analysis of either primary producers or primary consumers within the same ecosystem as the species of interest, necessitating additional sample collection, processing, and analysis that may not always be practical (Jennings and Warr, 2003; Fukumori et al., 2008). Even when baselines can be estimated in this manner, trophic omnivory and migration among habitats with different baselines can confound the interpretation of results (Post, 2002; Thompson et al., 2007).

Recent advances in the isotopic analysis of individual protein amino acids provide a means of overcoming these difficulties. The extent of nitrogen isotopic fractionation that occurs during dietary assimilation has been shown to vary among different amino acids, apparently due to differential rates of transamination and deamination reactions (McClelland and Montoya 2002; Chikaraishi et al. 2007; Popp et al. 2007). The amino acids glycine, lysine, methionine, phenylalanine, and serine fractionate very little with change in trophic level, and therefore are indicators of the isotopic baseline for the individual's basal resources, even in top-level predators. Because of this relatively conservative behavior, these compounds are commonly referred to as "source" amino acids. Conversely, alanine, aspartic acid, glutamic acid, leucine, isoleucine, and valine each fractionate sharply between diet resource and consumer, and are collectively referred to as "trophic" amino acids. The isotopic distance between unfractionated

source and fractionated trophic amino acids has recently come into use as an indicator of the trophic position occupied by individual organisms (Chikaraishi et al. 2007), providing freedom from the need for independent efforts to measure ecosystem baselines. As such, both trophic level (TL) and basal resource (baseline) nitrogen values can be determined from individual organisms with little or no a priori knowledge of the ecosystem from which they were collected.

This technique has been successfully applied to trophic determinations in systems where large-scale sampling regimes are difficult, such as the zooplankton community of the North Pacific gyre, Southern Ocean penguins, and deep-water Atlantic gorgonian corals (Hannides et al. 2009; Lorrain et al. 2009; Sherwood et al. 2011). The technique has also been used to detect changes in isotopic baselines underlying the diet of long-distance migrant fish (Popp et al. 2007). To our knowledge, this analytical approach has not previously been used to document ontogenetic trophic shifts within a single species, however.

In this study, compound-specific nitrogen isotopic analysis of muscle amino acids was performed on juvenile and adult silver perch (*Bairdiella chrysoura*) obtained from a single sampling event in Tampa Bay, Florida. The silver perch is a native member of the inshore and estuarine fish communities in the sampling area, where it subsists on a diet of zooplankton, benthic crustaceans and fishes. Previous stomach-content analysis of different length classes (Peebles and Hopkins, 1993) allowed independent comparison of stable-isotope results with traditional diet data. The purpose of the present research

is to use amino acid isotopes to characterize the trophic growth of an example fish species for which independent diet data are also available, and to evaluate and discuss different means of calculating isotope-based trophic level.

5.2: Methods:

Specimens were obtained during June 2011 by deploying a 3.5 mm mesh bag seine in seagrass meadows (1-2 m depth) within the interior of Terra Ceia Bay (27.554° N, 82.595° W), a semi-enclosed embayment that is part of the greater Tampa Bay estuary. Peak spawning by silver perch occurs during spring and early summer, with the two youngest year classes differing in length by approximately 80 mm during summer (Ayala-Pérez et al., 2006). Year classes from 2010 and 2011 were present in the Terra Ceia catch, with no length overlap between year classes. The 2010 year class was represented by five adult fish ranging 119-130 mm standard length (SL) and the 2011 year class was represented by 15 immature fish ranging 36-63 mm SL. Specimens were retained in iced seawater until return to the laboratory, where they were frozen at -50°C.

Upon thawing, individuals were weighed, measured, and a sample of muscle tissue was removed and oven-dried for 48 hours at 60^{o}C. After grinding to a fine powder, a 1 mg aliquot of each sample was removed for bulk isotopic determination via EA-IRMS.

For compound-specific isotopic analysis, a 5 mg aliquot of each tissue sample was digested in 2 mls of 6 N HCl under a nitrogen atmosphere at 100^{o} C for 24 hours. After digestion, acid was removed via evaporation under N_2 stream at 80^{o} and 100 µl of 0.01

molar norleucine solution was added as an internal standard. Digests were re-dissolved in 2 mls of 0.05N HCl, and amino acids were purified via ion-exchange chromatography, capitalizing on the fact that amino acids are retained on cation-exchange resins under acidic conditions but are released from these resins under basic conditions (Metges et al., 1996). Columns were assembled by packing 4 cm of Dowex 50WX8-400 cation-exchange resin into precombusted Pasteur pipettes plugged with quartz wool. Columns were preconditioned with 3 column volumes of 0.1 N HCl, then rinsed with 3 volumes of milli-Q water. Digests were then transferred onto the columns and rinsed with 3 column volumes of milli-Q water to flush non-amino acid contaminants. Finally, amino acids were eluted with 10 mls of 4 N NH_4OH. Eluents were dried under an N_2 stream at 50^oC and stored frozen until derivatization.

To enable gas chromatographic separation of amino acids, dried eluents were derivatized to N-acetyl isopropyl esters (Metges and Daenzer, 2000; Corr et al., 2007b). Samples were first esterifed in 2 mls of acidified isopropanol (2.8 N, 1:4 acetyl chloride:isopropanol) at 100^o C under N_2 for 60 minutes. Esterifying reagents were evaporated under an N_2 stream at 40^o and dried esters were rinsed with two sequential 500 uL aliquots of dichloromethane (Optima grade) to remove any residual acid solution.

Amino acid esters were acylated with 2 mls of acetic anhydride-triethylamine-acetone solution (1:2:5 by volume) under N_2 atmosphere at 60^o for 10 minutes. Derivatized amino acids were dried under an N_2 stream at room temperature and rinsed

with an additional two 500 ul aliquots of dichloromethane. Dried derivatives were re-dissolved in 1 ml of saturated NaCl-water solution and 1 ml of ethyl acetate and mixed vigorously. The organic phase containing the amino acids was transferred to a clean 4 ml vial and evaporated under N_2, while the aqueous phase was discarded. Finally, samples were re-dissolved in 500 uL of ethyl acetate and a 100 uL aliquot was removed for GC-IRMS analysis.

Samples were injected in splitless mode (3-4 µL injection volume) into an Agilent 6890 gas chromatograph linked to a Finnigan Delta Plus XL isotope ratio mass spectrometer via a GCC-II/III interface. Chromatographic separations were performed using a 60 meter x 0.25mm x 0.5 micron OV-1701 column suited to analysis of n-acetyl isopropyl amino acid esters (Meier-Augenstein, 2004). Injector temperature was 300^{o} C. The GC temperature program operated as follows: 80^{o} C initial, 2 minute hold; $40^{o}min^{-1}$ to 140^{o}; $2^{o}min^{-1}$ to 180^{o}; $5^{o}min^{-1}$ to 220^{o}; $15^{o}min^{-1}$ to 285^{o}, 12 minute final hold. Carrier gas supply was 1.5 ml min^{-1} in constant-flow mode.

Combustion of analyate peaks occurred in an oxidized pure metallic nickel tube (500 µM inner diameter) containing a copper/platinum wire braid and operated at a temperature of 1050^{o} C. No separate reduction reactor was required (Hilkert et al., 2009). Water and carbon dioxide were removed from the combustion products via a liquid nitrogen trap prior to entry of samples into the mass spectrometer.

System precision and accuracy were monitored via the presence of an internal standard (norleucine) in all samples as well as by the inclusion of a mixed amino acid

standard of known isotopic composition at the beginning and end of each run sequence. Measurement precision for the internal standard across the sample series was better than 0.5‰ (overall standard deviation = 0.4‰). Samples were run in duplicate and values presented are means. Agreement between individual sample replicates was generally better than 1‰ (0.6 ‰ for peaks > 250 mV mass 28 intensity).

Estimates of trophic position from amino acid nitrogen isotopic values are based on the degree of offset between source and trophic values. These estimates are often derived from single sets of amino acid pairs; phenylalanine-glycine and phenylalanine-glutamic acid comparisons are most commonly utilized for this purpose (McClelland and Montoya 2002; Loick , Gehre, and Voss 2007; Popp et al. 2007). A number of compounds, however, generally demonstrate quite similar behavior in this regard and can potentially be used interchangeably. Alanine, aspartic acid, and glutamic acid (grouped together as “trophic II” amino acids) have been suggested as the most reliable trophic indicators due to their roles in internal nitrogen cycling in eukaryotes (Hannides et al. 2009).

5.3: Results:

Nitrogen isotopic compositions of bulk muscle tissue suggested a dietary shift between juveniles and adults (Figure 5.1, Welsh’s t-test for unequal sample sizes, $P < 0.001$) in agreement with previous dietary reconstructions based on stomach-content analysis (Peebles and Hopkins, 1993). Compound-specific nitrogen isotopic compositions were successfully determined for the amino acids alanine, aspartic acid, glutamic acid,

glycine, leucine, lysine, phenylalanine, proline, serine, threonine, and valine. The $\delta^{15}N$ values for these compounds are presented in Table 5.1. Variation among different compounds within individuals was as high as 30‰. The additional amino acids isoleucine and methionine were also chromatographically resolvable, but low signal intensities and poor agreement between replicates led to them being excluded from further analysis. As expected, trophic amino acids (alanine, aspartic and glutamic acids, leucine, proline, and valine) were characteristically enriched relative to bulk isotopic compositions, while source amino acids (glycine, lysine, phenylalanine, serine, and threonine) were correspondingly depleted (Figure 5.2). Alanine, aspartic acid, and glutamic acid were consistently the most enriched in $\delta^{15}N$ while threonine was uniformly the most depleted.

Alanine, aspartic acid, and glutamic acid (trophic II amino acids) each showed a significant stepwise increase in $\delta^{15}N$ between juvenile and adult groups (Figure 5.3, Welsh's t-test, $P < 0.01$). In contrast, the $\delta^{15}N$ values for the source amino acids phenylalanine and lysine both showed no significant change with size (Figure 5.4). The amino acid threonine, interestingly, fractionated negatively with increasing size (Figure 5.5).

Of the remaining compounds, both glycine and serine grouped with source amino acids and showed minimal trophic enrichment, although each demonstrated greater variability than did phenylalanine and lysine. Leucine and valine showed clear enrichment in ^{15}N among adults relative to juveniles, as expected. Proline, however, did

not, despite typically being classified as a trophic-indicating amino acid (McClelland and Montoya, 2002).

5.4: Discussion

The silver perch consumes a varied diet that is strongly dominated by planktonic calanoid copepods at sizes up to 50 mm, after which individuals transition to fishes and benthic mysids, with benthic gammaridean amphipods serving as important prey during the transition (Table 5.2, Peebles 2005). The transition appears to be centered at approximately 80 mm SL. This transition is consistent with both the bulk and compound-specific isotopic results of this study.

The patterns of fractionation seen in the "trophic II" amino acids (alanine, aspartic acid, and glutamic acid) show a significant shift between juveniles and adults, implying the latter occupy a fractionally higher trophic level. In each case, however, it is important to note that the relationship is not linear with size but rather appears to exist as a step function. Regressions of each amino acid isotopic composition against standard length within the entire juvenile population uniformly produce slopes that are not significantly different from zero ($p > 0.05$). In this regard, all juveniles—regardless of size—occupy the same trophic position. The transition to the adult diet appears to take place during the gap in lengths in the present data (likely near 80 mm SL, as indicated by diet data).

In order to calculate estimates of trophic position from amino acid isotopes, it is necessary to take into account the fractionation between source and trophic amino

acids during dietary assimilation as well as the fractionation between the compounds of interest in the primary producers themselves. Several formulations have been put forward to do so. Chikaraishi et al. (2009) surveyed a variety of organisms both from natural environments and culture studies and derived an equation for trophic position based on glutamic acid and phenylalanine, with an estimated trophic fractionation of +7.6‰ for glutamic acid, no trophic fractionation for phenylalanine, and an initial difference ($\beta_{glu\text{-}phe}$) of +3.4‰ within primary producers, where

$$TL_{glu\text{-}phe} = [(\delta^{15}N_{glu} - \delta^{15}N_{phe} - 3.4)/7.6] + 1$$

This is a modification of the initial estimator established by McClelland and Montoya (2002), who used the same amino-acid pair and suggested a trophic fractionation for glutamic acid of +7‰ across a single trophic step between phytoplankton and zooplankton.

A very similar formula was put forward by Hannides et al. (2009). Here, the assumed trophic fractionation of glutamic acid was also +7‰, but $\beta_{glu\text{-}phe}$ was +4‰:

$$TL_{glu\text{-}phe} = [(\delta^{15}N_{glu} - \delta^{15}N_{phe} - 4)/7] + 1$$

Within the analytical error for $\delta^{15}N$ measurements for amino acids, these two equations are essentially identical. Hannides et al. also supplied an alternative equation using the glycine-phenylalanine pair, as had been used by Popp et al. (2007) for estimating trophic position of Pacific yellowfin tuna, *Thunnus albacares*:

$$TL_{glu\text{-}gly} = [(\delta^{15}N_{glu} - \delta^{15}N_{gly})/7] + 1$$

When the latter two equations were applied to the present silver perch data (Table 5.1), there was close agreement is the estimated TL difference between juveniles and adults (both estimated ~0.5 TL difference), yet $TL_{glu\text{-}phe}$ appeared to underestimate actual TL. Stomach-content data indicate smaller silver perch are secondary consumers, primarily subsisting on planktonic copepods, which would place them at approximately TL 3.0-3.5, depending on the extent of omnivory in the copepods (Conley and Turner, 1985). This is inconsistent with an estimated juvenile $TL_{glu\text{-}phe}$ of 2.4, which would suggest a diet containing a substantial proportion of primary producers. In contrast, the glutamic acid-glycine estimate ($TL_{glu\text{-}gly}$) indicated a more reasonable mean juvenile TL of 2.9 and an adult value of 3.3, which are in much closer agreement with the diet data.

There are several possible causes for this disparity. First, the diets of the fish in the present study may have differed from those surveyed for stomach contents. There are infrequent reports of juvenile silver perch consuming sediments (and presumably associated benthic microalgae) and vascular plant remains, but these are generally discounted as being insignificant contributors to the overall diet (Stickney et al., 1975).

There is also the possibility of subtle differences in measurement caused by methodological nuances in the derivatization scheme and GC parameters used in analysis. The results for amino acids standards of known composition, however, were both precise and accurate, indicating procedural artifacts would have been very small. Nevertheless, rigorous inter-calibrations between different methodologies and

laboratories would be useful in the future, but work in this area has not yet reached sufficient density to merit this type of effort.

The coefficients used in defining $TL_{glu\text{-}phe}$ are empirical and were derived from the existing body of amino acid nitrogen isotope measurements in literature, which are still fairly sparse (Chikaraishi et al. 2009). While this relationship is the most robust indicator of trophic position yet available across a broad range of species, it may not be the optimal one for fish. A re-evaluation of the Popp et al. (2007). *Thunnus* data produced an offset between trophic position estimates similar to the one seen for silver perch in this study (Table 5.3). Further research is needed into the exact determinants of the magnitudes of compound-specific trophic fractionations to resolve this issue.

Finally, the patterns of fractionation seen in threonine also merit further attention. Owing to problems with chromatographic resolution, threonine is not commonly reported in compound-specific amino acid studies. The available data suggest negative trophic fractionation does, in fact, occur widely in this compound (Hare et al. 1991; Hannides et al. 2009). Threonine is one of only two protein amino acids for which a transaminase is not known to exist. This likely has some bearing on its unusual behavior, since transamination is proposed as an explanation for the positive fractionation seen in trophic amino acids. It appears that this compound, then, could be used as an independent measure of trophic position by comparing its composition to non-trophic fractionating compounds. Further information on the extent and variability of its trophic fractionation would be required, however.

The silver perch's ontogenetic diet shift is independently warranted by the improving optimality of larger prey (mysids and fish) that accompanies growth-related gape increases, but the shift may also be mandated by seasonal trends in prey availability. Copepod abundance in Tampa Bay decreases markedly during some, but not all, winters (Hopkins, 1977; Peebles, 1996), such that silver perch individuals that were spawned late in the summer may experience reduced survival or fitness if they cannot advance to larger prey before the onset of winter, as was observed by Post (2003) for largemouth bass at more temperate latitudes. The lack of significant trends between trophic amino acids and juvenile fish length (Figure 5.3) suggests the shift is not gradual and continuous.

5.5: Conclusions

Amino acid nitrogen isotope measurements showed a clear ontogenetic trophic shift between juvenile and adult silver perch, in good agreement with previous stomach-content-based measures from the same region. The magnitude of this change was approximately half a trophic step, similar to the difference suggested by bulk nitrogen measurements. The "trophic II" amino acids alanine, aspartic acid, and glutamic acid were equally valid indicators of trophic position, while phenylalanine and lysine were the most faithful indicators of ecosystem isotopic baseline. Threonine was found to fractionate negatively with increasing trophic position, for reasons that are not readily apparent but are in agreement with other studies.

Estimates of trophic level from the isotopic distance between source and trophic amino acids varied according to the formula used, but uniformly captured the extent of trophic-level difference between juveniles and adults. Trophic determinations using the glycine-phenylalanine method of Popp et al. (2007) appeared to produce the most accurate estimates for this species, as was also the case with *Thunnus albacares*, and may possibly be true of fish in general. While there is still a need for refinement in the understanding of the underlying dynamics of amino acid nitrogen isotopic fractionation during trophic transfers, the method seems well suited to rapid assessments of ontogenetic diet shifts in fishes.

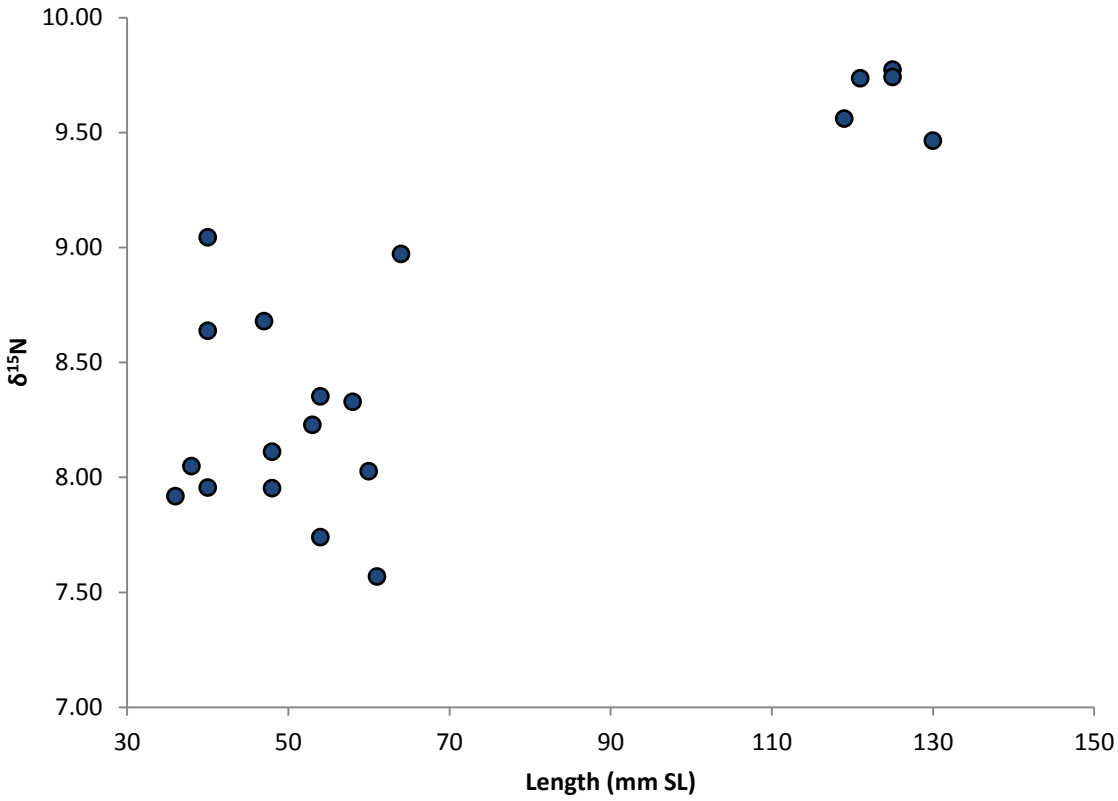


Figure 5.1: Silver perch bulk $\delta^{15}N$ values vs. standard length.

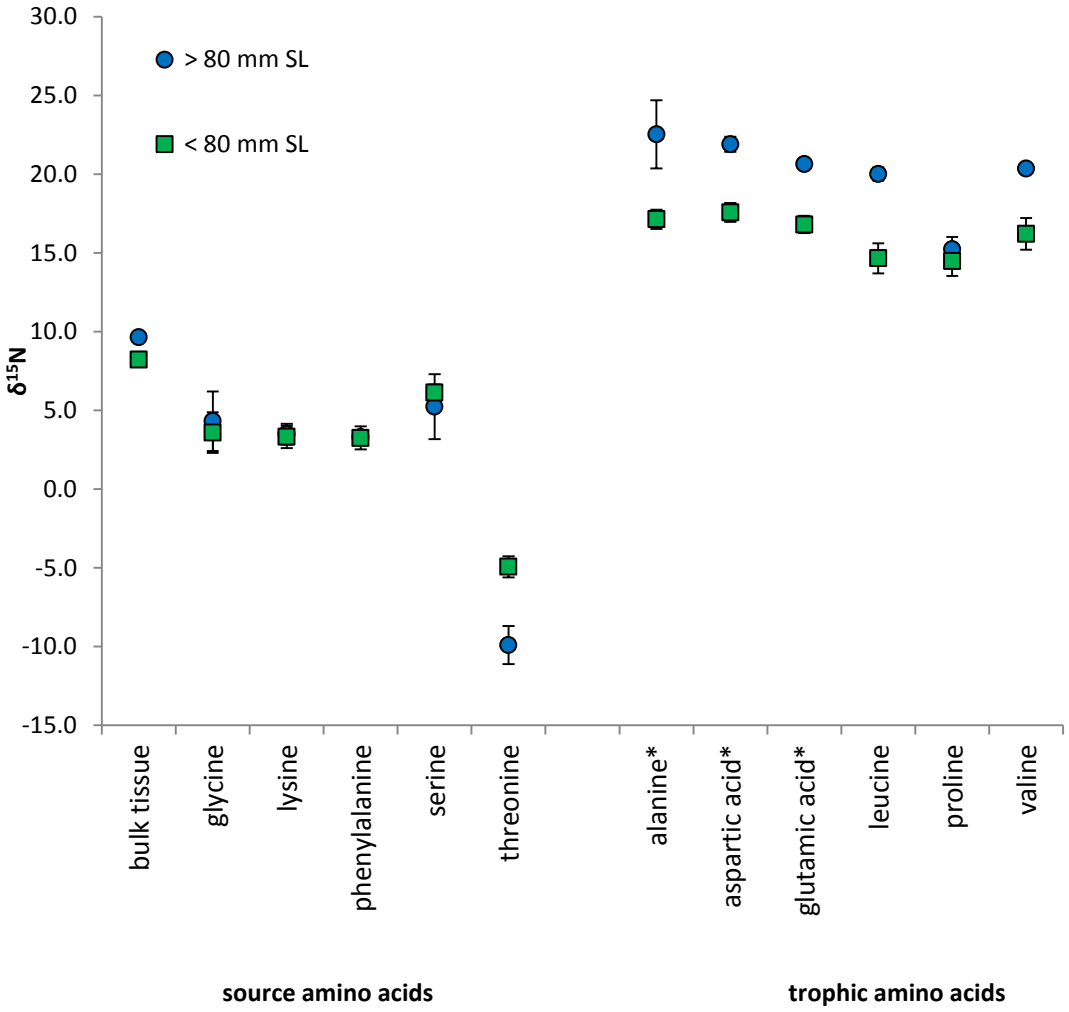


Figure 5.2: Mean amino acid $\delta^{15}N$ values for juvenile (<80 mm SL) and adult (>80 mm SL) silver perch. Error bars indicate standard deviations within each group. Bulk $\delta^{15}N$ values are presented for comparison. "Trophic II" amino acids (Hannides et al. 2009) are marked with an asterisk.

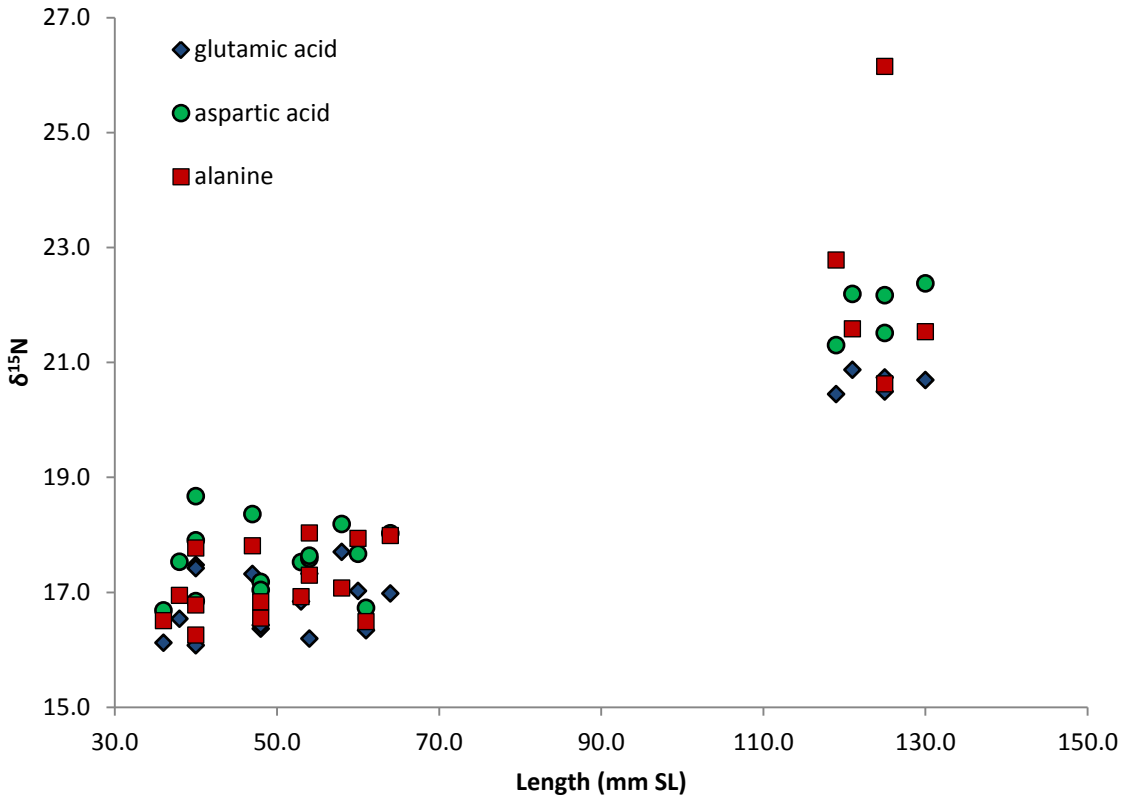


Figure 5.3: Silver perch $\delta^{15}N$ values for trophic-indicating amino acids vs. standard length

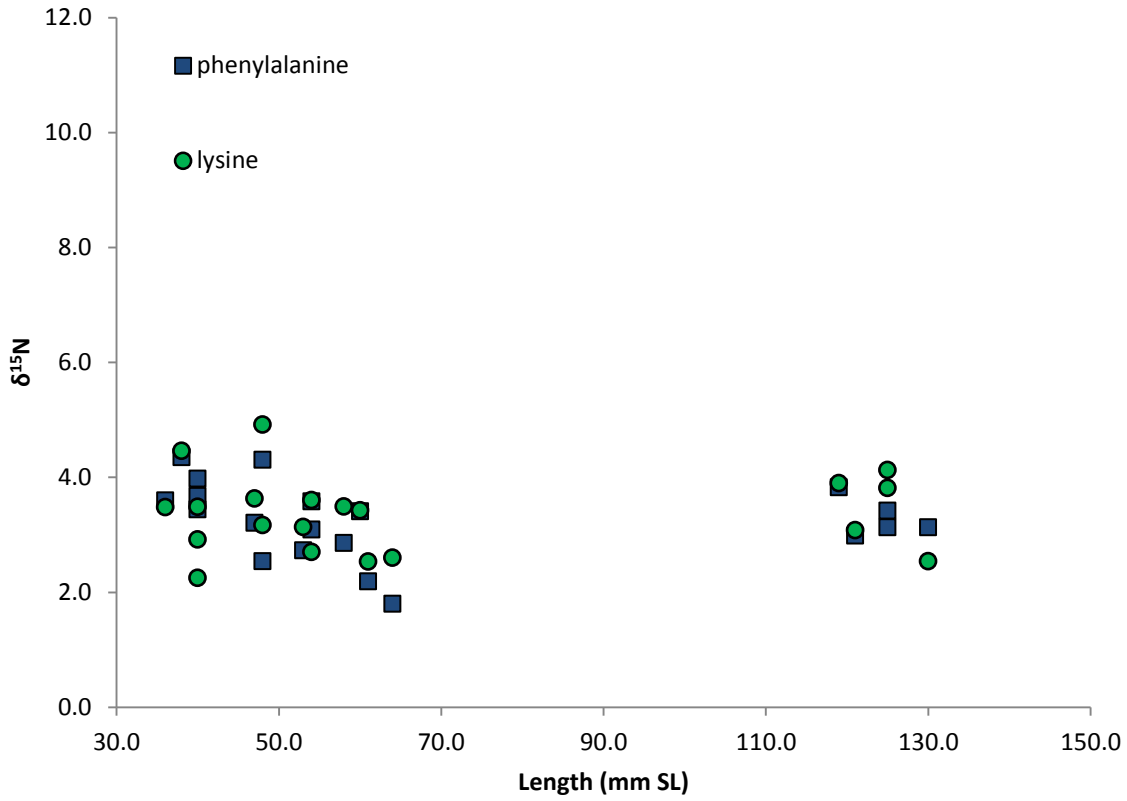


Figure 5.4: Silver perch $\delta^{15}N$ values for source-indicating amino acids vs. standard length

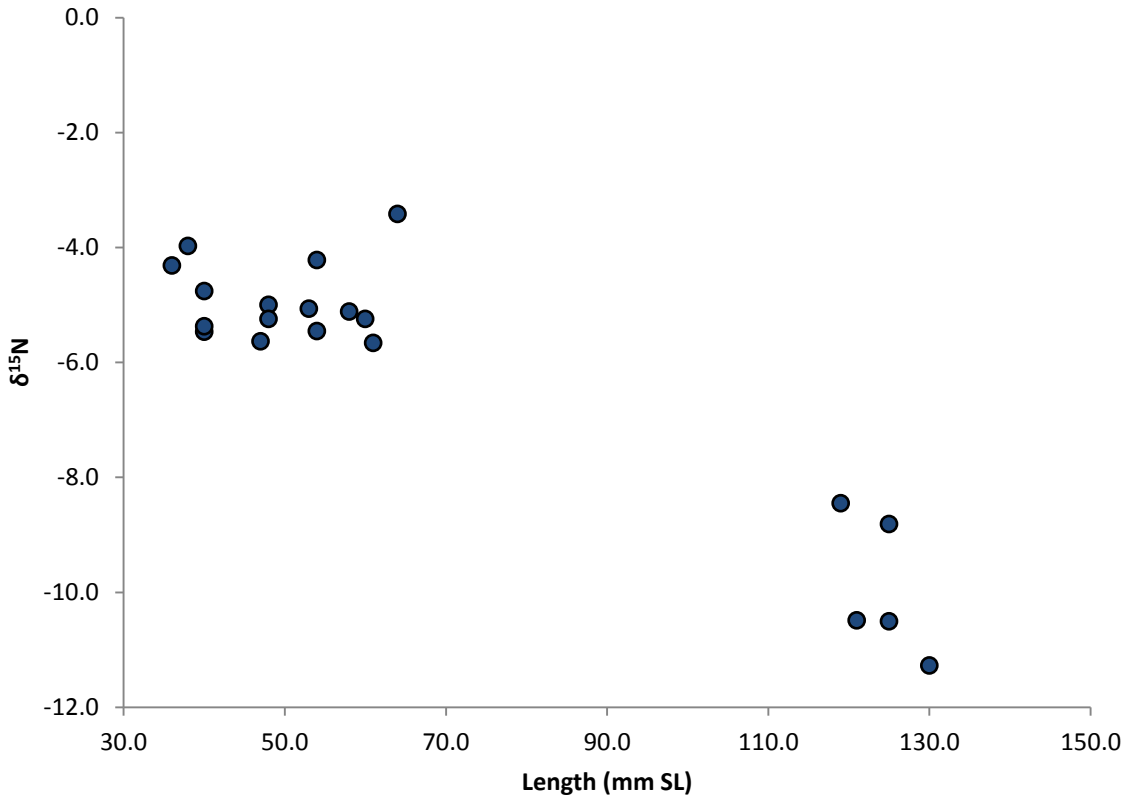


Figure 5.5: Silver perch $\delta^{15}N$ values for the amino acid threonine vs. standard length.

Table 5.1: Amino acid $\delta^{15}N$ values for individual silver perch, listed by increasing standard length. Asterisks denote "trophic II" amino acids that are thought to produce the most consistent trophic fractionations (Hannides et al. 2009). Bulk muscle tissue $\delta^{15}N$ values are listed for comparison. Amino acid-derived estimates of trophic level based on (glutamic acid - phenylalanine) and (glutamic acid - glycine) are compared in the last two columns (see text, Hannides et al. 2009; Chikaraishi et al. 2009).

		source amino acids					trophic amino acids							
length (mm SL)	bulk $\delta^{15}N$	glycine	lysine	phenylalanine	serine	threonine	alanine*	aspartic acid*	glutamic acid*	leucine	proline	valine	trophic level (glu-phe)	trophic level (glu-gly)
36	7.9	3.9	3.5	3.6	6.4	-4.3	16.5	16.7	16.1	13.6	14.5	15.2	2.2	2.7
38	8.0	5.9	4.5	4.4	6.2	-4.0	16.9	17.5	16.5	15.0	15.1	15.6	2.2	2.5
40	8.0	2.7	3.5	3.4	5.7	-4.8	16.3	16.8	16.1	14.7	12.9	15.6	2.2	2.9
40	9.0	3.0	2.2	4.0	6.1	-5.5	17.8	18.7	17.5	16.1	15.9	17.7	2.4	3.1
40	8.6	2.8	2.9	3.7	5.0	-5.4	16.8	17.9	17.4	15.6	15.0	16.6	2.4	3.1
47	8.7	3.9	3.6	3.2	6.7	-5.6	17.8	18.4	17.3	15.5	14.3	16.5	2.4	2.9
48	8.0	2.6	3.2	4.3	6.0	-5.0	16.5	17.2	16.4	14.3	13.9	15.1	2.2	3.0
48	8.1	3.8	4.9	2.5	5.6	-5.2	16.8	17.0	16.4	14.1	13.8	15.5	2.4	2.8
53	8.2	2.5	3.1	2.7	6.7	-5.1	16.9	17.5	16.8	14.7	15.3	16.3	2.4	3.0
54	7.7	3.7	3.6	3.6	5.7	-5.5	17.3	17.6	16.2	14.5	13.8	15.9	2.2	2.8
54	8.4	6.1	2.7	3.1	7.0	-4.2	18.0	17.6	17.3	15.4	14.5	16.4	2.5	2.6
58	8.3	2.7	3.5	2.9	6.2	-5.1	17.1	18.2	17.7	15.0	14.6	17.2	2.5	3.1
60	8.0	2.9	3.4	3.4	6.2	-5.2	17.9	17.7	17.0	13.5	15.1	15.7	2.4	3.0
61	7.6	1.9	2.5	2.2	6.0	-5.7	16.5	16.7	16.3	12.6	12.8	15.2	2.4	3.1
64	9.0	5.6	2.6	1.8	6.7	-3.4	18.0	18.0	17.0	15.6	15.8	18.7	2.6	2.6
119	9.6	7.3	3.9	3.8	6.9	-8.5	22.8	21.3	20.4	19.7	15.8	20.6	2.8	2.9
121	9.7	3.0	3.1	3.0	5.3	-10.5	21.6	22.2	20.9	20.1	14.8	20.4	3.0	3.6
125	9.8	5.1	4.1	3.4	5.9	-8.8	20.6	21.5	20.5	19.8	14.4	20.4	2.9	3.2
125	9.7	3.4	3.8	3.1	6.4	-10.5	26.1	22.2	20.7	20.7	16.3	20.5	2.9	3.5
130	9.5	2.8	2.5	3.1	1.7	-11.3	21.5	22.4	20.7	19.8	14.9	19.8	2.9	3.6

Table 5.2: Index of relative importance (IRI) of major constituents of silver perch diet by size class (Peebles and Hopkins, 1993). IRI is an aggregate function of the frequency of occurrence, estimated volume, and abundance of a given prey item in stomach contents intended to provide a weighted value of dietary importance(Pinkas et al., 1971). Juvenile size classes are dominated by copepods and amphipods, while adult diets consist largely of fishes and mysids. Number of individuals surveyed in each size class listed in parentheses.

	length class (mm SL)							
item	21-30 (n = 93)	31-40 (n = 93)	41-50 (n = 80)	51-60 (n = 87)	61-70 (n = 50)	71-80 (n = 27)	81-90 (n = 20)	91-160 (n = 57)
calanoid copepods	116	94	49	16	7			
gammaridean amphipods	1	6	8	22	23	6	9	<1
mysids	2	6	1	5	3	37	10	51
shrimps	<1	2	2	5	3	1	2	3
fishes				2	4	11	26	3

Table 5.3: Trophic calculations for *Thunnus albacares* isotopic compositions from Popp et al. (2007). The glutamic acid-phenylalanine method consistently underestimates trophic level relative to the glutamic acid-glycine method for this species.

latitude	8° 13.8′ S	1° 10.8′ S	10° 31.8′ N	13° 45.0′ N	24° 15.0′ N
bulk	10.4	10.9	13.5	14.7	15.6
glutamic acid	27.3	23.4	24.7	29.2	30.6
phenylalanine	2.7	2.7	3.2	5.3	7.5
glycine	1.7	-2.3	2.8	7.2	7.5
$TL_{glu\text{-}gly}$	4.7	4.7	4.1	4.1	4.3
$TL_{glu\text{-}phe}$	3.9	3.4	3.5	3.8	3.7
offset	0.7	1.3	0.6	0.3	0.6

Chapter 6: Effects of nutritional condition on amino acid-based estimates of trophic position in the Bay Anchovy (*Anchoa mitchilli*) from the Alafia River, Florida

6.1: Introduction

Nitrogen stable isotopic analysis of tissue samples has been a mainstay of trophic ecological research for many years due to the predictable increase observed in $\delta^{15}N$ up food chains. The light isotope of nitrogen is preferentially excreted during metabolic processing, leading to a stepwise enrichment of approximately 2 - 4‰ per trophic level in organisms (Minagawa and Wada 1984; Peterson and Fry 1987; Vander Zanden and Rasmussen 2001).

A shortcoming of this technique arises from the fact that it is by definition a relative measure. While different organisms within a single food chain can be assigned an appropriate trophic position in this way, any comparison between systems requires knowledge of the local isotopic baseline values. Different environments have nutrient sources with widely varying $\delta^{15}N$ values to which any trophic fractionation is ultimately added (Cabana and Rasmussen 1996; Vander Zanden and Rasmussen 1999). Organisms occupying equivalent trophic positions in different ecosystems will therefore not have any predictable relationships in $\delta^{15}N$, making comparisons between such samples fruitless absent knowledge of underlying inter-system isotopic background differences.

Compound-specific isotopic analysis of amino acid nitrogen is a recently developed method to address this problem by deriving data on both ecosystem baseline $\delta^{15}N$ values and the degree of trophic fractionation from a single sample. Amino acids that participate widely in exchange of nitrogen through transamination reactions, including alanine, aspartic and glutamic acids, leucine, proline, and valine, ("trophic" amino acids) have been shown to fractionate heavily between trophic levels up food chains. In contrast, those amino acids that rarely undergo transamination, including glycine, lysine, phenylalanine, serine, and threonine, ("source" amino acids) retain the isotopic composition of their original nutrient precursors (McClelland and Montoya, 2002). The relative distance in $\delta^{15}N$ values between the two groups therefore provides an internal estimate of trophic position without the need for knowledge of a sample's environmental history.

The results of this differential fractionation have been formalized into mathematical relationships for performing single-sample trophic determinations. Typically, differences in isotopic composition between either representative compounds or average values for each group are compared to fractionation factors derived from culture experiments to make assignments of trophic position. Various combinations of compounds have been employed, including glutamic acid – glycine, glutamic acid – phenylalanine, and mean trophic – mean source (Popp et al. 2007; Chikaraishi et al. 2007; Hannides et al. 2009). Of these, the formula developed by Chikaraishi was developed using the broadest available set of empirically measured trophic fractionations and is shown here as

representative of the general format that all of these relationships follow (Chikaraishi et al. 2009):

$$TL_{glu\text{-}phe} = [(\delta^{15}N_{glu} - \delta^{15}N_{phe} - 3.4)/7.6] + 1$$

The robustness of the coefficients employed is a major concern, as deviations in the actual trophic fractionation from the assumed values will lead to potentially large errors in assigned trophic positions. Despite this, due to the relative novelty of this area of research, the values presented by Chikaraishi were compiled from only approximately thirty species, largely raised in culture, and weighted heavily towards planktonic organisms. It therefore remains unknown how reliable they will prove in the face of real-world environmental variability.

It has been shown that the bulk nitrogen isotopic composition of animal tissue samples is influenced both by the quantity and quality of an organism's food supply. Fasting in birds was found to result in modestly elevated ^{15}N values both in wild populations and in culture experiments (Hobson et al., 1993). Similarly, manipulations of diet in zooplankton culture showed that the extent of trophic fractionation was tightly correlated to the nitrogen content of the feed supplied, with fractionation increasing sharply as C:N ratio increased (Adams and Sterner, 2000). The mechanism for this phenomenon is thought to be increased internal nitrogen recycling during periods of nutrient stress: essentially, an organism with a negative net nitrogen supply is "preying" on itself through catabolic processes (Gannes et al., 1998). Despite these effects observed in bulk measurements, the impact of these processes on amino acid-specific

isotopic values and on relationships between $\delta^{15}N$ values of different compounds is currently unknown.

Here, we test for differences in compound-specific nitrogen isotopic fractionation between cohorts of bay anchovy, *Anchoa mitchilli*, exposed to differing nutrient regimes in the Alafia River, FL. This species is endemic in Florida estuaries and tidal rivers, where its distribution is governed by spatial and temporal patterns of prey availability (Peebles et al., 2007). In the Alafia River, heavy summer seasonal rainfall causes a seaward movement of *A. mitchilli* into the vicinity of a shipping channel subject to periodic hypoxia. This pattern constrains access to food resources in the summer months, leading to annual bouts of nutritional stress or starvation in the area. This occurrence has been routinely observed over more than a decade of sampling of the local fish community (Ernst Peebles, personal communication). By comparing patterns of amino acid $\delta^{15}N$ values obtained from dry season (healthy) and wet season (stressed) collections of anchovies, this study tests the robustness of compound-specific fractionation factors to variations in nutritional state of the sample organisms in both fresh and archived (formalin-isopropanol fixed) specimens.

6.2: Methods

All samples of *A. mitchilli* used in this study were collected as part of a long-term monitoring program of the forage-fish community in the Alafia River, FL. Fish were captured with a fine-mesh trawl and stored on ice until return to the laboratory. 2011 samples were taken in May (dry season) and July (wet season) and in both cases were

processed immediately as described below. Fixed samples were obtained from archived collections held at the USF College of Marine Science taken in the same months in both 2006 and 2001.

For each 2011 collection period, 25 randomly selected individuals were weighed and measured (standard length) to obtain an estimate of the population length-weight distribution. Length-weight regression models were developed for each collection using the Statgraphics software package to show seasonal changes in mean nutritional state. Twelve individuals were then randomly chosen from each sample for isotopic analysis. For each of these, a sample of muscle tissue was removed and oven-dried for 48 hours at 60^{o}C. Dried tissue was ground to fine powder in an agate mortar and pestle, and a 1 mg aliquot of each was removed for bulk isotopic analysis via EA-IRMS using a CE Instruments NA 2500 elemental analyzer linked to a Delta Plus XL isotope ratio mass spectrometer.

Fixed samples were treated similarly to the previously described methods with two differences. The number of individuals used for isotopic analysis was six rather than twelve, and length-weight values were not calculated due to small numbers of individuals present as well as visible differences in preservational state. Bulk and compound-specific isotopic analysis were performed identically to the 2011 samples.

For compound-specific isotopic analysis, a 5 mg aliquot of each tissue sample was digested in 2 mls of 6 N HCl under a nitrogen atmosphere at 100^{o} C for 24 hours. After digestion, acid was removed via evaporation under N_2 stream at 80^{o} and 100 uL of 0.01

molar norleucine solution was added as an internal standard. Digests were re-dissolved in 2 mls of 0.05N HCl and amino acids were purified via ion-exchange chromatography on cation-exchange columns. Under acidic conditions, amino acids are retained on the columns, allowing separation from any other residual material in the digests. Subsequent to the elution of any undesired compounds, amino acids can be released by flushing with strong base (Metges et al., 1996). Columns were assembled by packing 4 cm of Dowex 50WX8-400 cation-exchange resin into precombusted Pasteur pipettes plugged with quartz wool. Columns were preconditioned with 3 column volumes of 0.1 N HCl, then rinsed with 3 volumes of milli-Q water. Digests were then transferred onto the columns and rinsed with 3 column volumes of milli-Q water to flush non-amino acid contaminants. Finally, amino acids were eluted with 10 mls of 4 N NH_4OH. Eluents were dried under an N_2 stream at 50^{o}C and stored frozen until derivatization.

Purified amino acids were derivatized to N-acetyl isopropyl esters to facilitate chromatographic separation (Metges and Daenzer, 2000; Corr et al., 2007b). Samples were first esterifed in 2 mls of acidified isopropanol (2.8 N, 1:4 acetyl chloride:isopropanol) at 100^{o} C in sealed vials under an N_2 atmosphere for 60 minutes. After cooling to room temperature, esterifying reagents were evaporated under an N_2 stream at 40^{o} and dried esters were rinsed with two sequential 500 uL aliquots of dichloromethane (Optima grade) to remove any residual acid solution.

Amino acid esters were acylated with 2 mls of acetic anhydride-triethylamine-acetone solution (1:2:5 by volume) under N_2 atmosphere at 60^{o} for 10 minutes.

Derivatized amino acids were dried under an N_2 stream at room temperature and rinsed with an additional two 500 ul aliquots of dichloromethane. Dried derivatives were re-dissolved in 1 ml of saturated NaCl-water solution and 1 ml of ethyl acetate and mixed vigorously. The organic phase containing the amino acids was transferred to a clean 4 ml vial and evaporated under N_2, while the aqueous phase was discarded. Finally, samples were redissolved in 500 uL of ethyl acetate and a 100 uL aliquot was removed for GC-IRMS analysis.

Samples were injected in splitless mode (3-4 µL injection volume) into an Agilent 6890 gas chromatograph linked to a Finnigan Delta Plus XL isotope ratio mass spectrometer via a GCC-II/III interface. Chromatographic separations were performed using a 60 meter x 0.25mm x 0.5 micron OV-1701 column suited to analysis of n-acetyl isopropyl amino acid esters (Meier-Augenstein, 2004). Injector temperature was 300^{o} C. The GC temperature program operated as follows: 80^{o} C initial, 2 minute hold; 40^{o} per minute to 140^{o}; 2^{o}/minute to 180^{o}; 5 ^{o}/minute to 220^{o}; 15^{o}/minute to 285^{o}, 12 minute final hold. Carrier gas supply was 1.5 mls per minute, constant flow mode.

Combustion of analyate peaks occurred in an oxidized pure metallic nickel tube (500 µM inner diameter) containing a copper/platinum wire braid and operated at a temperature of 1050^{o} C. No separate reduction reactor was required (Hilkert et al., 2009). Water and carbon dioxide were removed from the combustion products via a liquid nitrogen trap prior to entry of samples into the mass spectrometer.

System precision and accuracy were monitored via the presence of an internal standard (norleucine) in all samples as well as by the inclusion of a mixed amino acid standard of known isotopic composition at the beginning and end of each run sequence. Measurement precision for the internal standard across the sample series was better than 0.5‰ (overall standard deviation = 0.4‰). Samples were run in duplicate and values presented are means. Agreement between individual sample replicates was generally better than 1‰ (0.6 ‰ for peaks > 250 mV mass 28 intensity).

6.3: Results

Bay anchovies collected in May 2011 had a mean length of 22.7 mm and a mean mass of 125.2 mg. The July 2011 collection means were 23.0 mm and 106.5 mg, respectively (Table 6.1). There was no significant difference in lengths between samples, while July samples had significantly lower weights (T-test, P = 0.046), in keeping with the expectation for nutritional stress during the local rainy season. Bulk tissue nitrogen isotopic values were lower for July samples, but this difference was not significant.

Length-weight regression models were calculated for each sample period using a square-root Y model of the form $Y = (a + b*X)^2$ fit using the Statgraphics software package (Figures 6.1 and 6.2, respectively). This model has been found to best fit the long-term monitoring series for Bay Anchovy at this location (Ernst Peebles, personal communication). Comparison of the calculated regression equations showed them to differ significantly in slope ($p < 0.05$), further demonstrating a difference in mean condition between months.

For freshly collected specimens, amino acid $\delta^{15}N$ values ranged from -4.5 to 25.5, with source amino acids universally depleted relative to trophic ones (Tables 6.2 & 6.3; Figure 6.3). Glutamic acid and alanine had the most enriched values, while threonine was markedly depleted relative to all other compounds. Despite the differences in physical condition between the two samples, amino acid nitrogen isotopic compositions were very similar. Trophic fractionation (as measured by $\delta^{15}N_{glutamic\ acid} - \delta^{15}N_{phenylalanine}$) did not differ significantly between samples (Student's T, $p = 0.11$). July 2011 samples were slightly ^{15}N-depleted relative to May samples in all compounds except alanine and proline, suggesting the possibility of a small shift in ^{15}N baseline between sampling periods, but this did not appear related to nutritional condition. This paralleled the small depletion seen in bulk nitrogen isotopic values.

Fixed samples from both 2006 and 2001 occupied the same range of amino acid δ^{15} values as the 2011 samples, although chromatographic performance in their analysis was worse (Figures 6.4 and 6.5, respectively) . Proline and threonine co-eluted and could not be separated, while glycine and alanine were sometimes also unresolvable, especially in the older samples (Table 6.4). While direct comparisons of individual weights between seasons were not possible due to visible differences in preservation, no clear difference in isotopic fractionation was evident between wet and dry seasons in either of the years sampled.

6.4: Discussion

As expected, the mean condition of anchovies collected in the July 2011 sample was worse (as measured by length-weight regressions) than the corresponding May 2011 value. This is strongly suggestive of starvation and net loss of nitrogen via protein catabolism in the intervening period. Despite this, there were only modest differences in amino acid $\delta^{15}N$ values and no distinguishable changes in patterns of fractionation between source and trophic amino acids between samples. This suggests that the extent of fractionation between amino acids within an organism may be relatively robust and fairly insensitive to changes in condition. If proven, this would reinforce the utility of amino acid-based techniques in studies of trophic ecology. There are, however, substantial caveats in the interpretation of the current results.

While drawn from the same habitat with a resident population of *A. mitchilli*, there is no way of knowing with certainty if the two collections shared identical life histories. Loss of access to preferred food resources at the start of the rainy season could easily have led to consumption of less desirable materials in the interval between sample collections; as such the effective isotopic baseline could have changed over that period. The relatively worse condition of the July specimens only indicates a net energy deficiency, but does not prove absence of any feeding.

The Alafia River is a highly eutrophic system. Presumably this means most available prey items within it are relatively nitrogen-rich. Variability in bulk isotopic trophic fractionation factors have been shown to be strongly related to the nitrogen content of

the diet rather than to overall energy supply, with fractionation sharply increasing in diets with high C:N ratios (Adams and Sterner, 2000). The lack of a significant difference in bulk $\delta^{15}N$ compositions between the two collections argues against this mechanism coming into play in this case. Presumably, if starvation and catabolism manifested themselves in the same way as nitrogen limitation, the July specimens would have had noticeably elevated bulk $\delta^{15}N$ values. This was not the case. Since bulk nitrogen isotopic values are driven by changes in amino acid $\delta^{15}N$, it is therefore perhaps unsurprising that no significant differences were observed on the compound-specific level, either. It does not, however, address the fundamental question of the compound-specific basis for variations in bulk nitrogen isotope fractionation factors due to changes in diet quality reported elsewhere.

Disentangling these problems will require experiments where more parameters can be accurately controlled. Culture studies seem like an obvious solution to this problem, and would allow more thorough understanding of the effects of both nutrient- and energy- deficient diets (low N and low C intakes, respectively) on compound-specific isotopic fractionation. To our knowledge, such experiments have not yet been done, and would seem a fruitful area of future research.

Preserved samples from five and ten years prior to the current collections showed strikingly similar patterns of amino acid $\delta^{15}N$, suggesting relatively few changes in the nutrient dynamics of the Alafia River system over that time and confirming the effectiveness of the technique for analysis of fixed organic matter. While there have

been previous reports of successful amino acid $\delta^{15}N$ analysis of formalinated samples, they were restricted to physically small specimens such as zooplankton (Hannides et al. 2009). The larger body size of the anchovies used here may explain the relatively worse performance of the derivatization and subsequent chromatographic separation with these materials. While speculative, it would seem likely that preservation was incomplete on the molecular level, leading to the selective degradation of several amino acids. The larger number of unresolvable peaks in the older samples is suggestive of this. Despite this, the ability to make accurate trophic determinations was not compromised in either case, confirming that fixed samples in archived collections are amenable to analysis of this sort.

6.5: Conclusions

Trophic level assessments based upon patterns of amino acid $\delta^{15}N$ in this study did not differ between samples with differing length-weight relationships, suggesting that the magnitude of differences in source-trophic amino acid combinations are not sensitive to dietary stress. This, in turn, suggests that estimates of trophic position obtained based upon these relationships are not impacted by starvation or dietary deficiencies. Within the boundaries of the caveats previously discussed, this helps strengthen the utility of amino acid-based trophic determinations.

The comparison between freshly caught and archived, fixed samples showed that similar results could be obtained from both. While previous researchers had demonstrated that successful amino acid $\delta^{15}N$ analysis could be performed on

formalinated plankton samples, demonstrating the effectiveness of this technique in larger organisms such as fish greatly expands the range of samples available for ecological reconstructions.

Further research is needed to fully understand the effects of limitations in both carbon and nitrogen supply on amino acid-specific nitrogen isotopic fractionation factors. Controlled culture experiments will be required to gain further insights in this area.

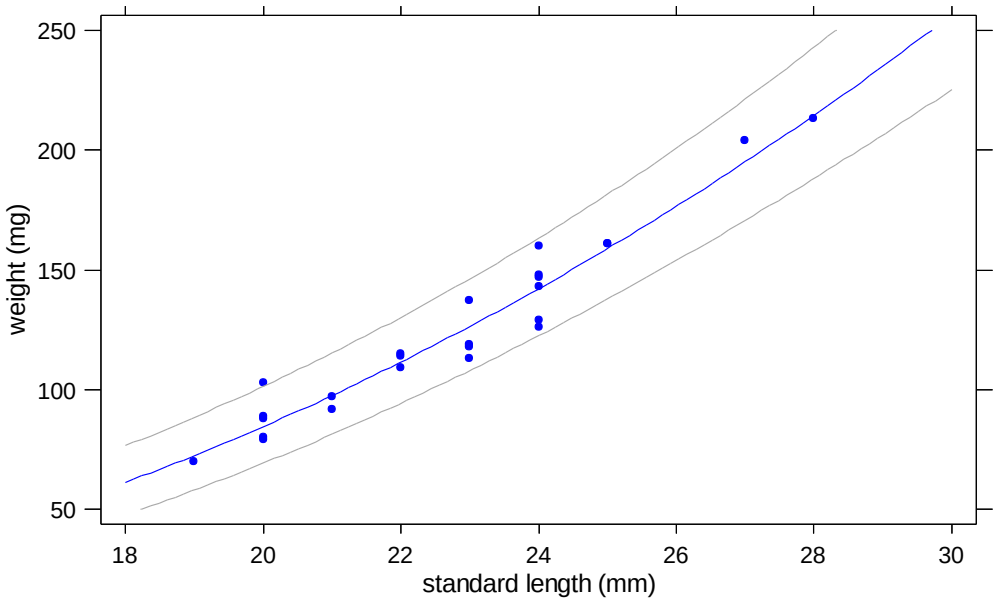


Figure 6.1: Square-root Y regression of standard length vs. weight for Alafia River *Anchoa mitchilli*, May 2011. Grey lines indicate limits of prediction for the equation. The regression line is defined by the relationship weight = $(-4.41 + 0.68 * \text{length})^2$. Adjusted $R^2 = 94.0\%$.

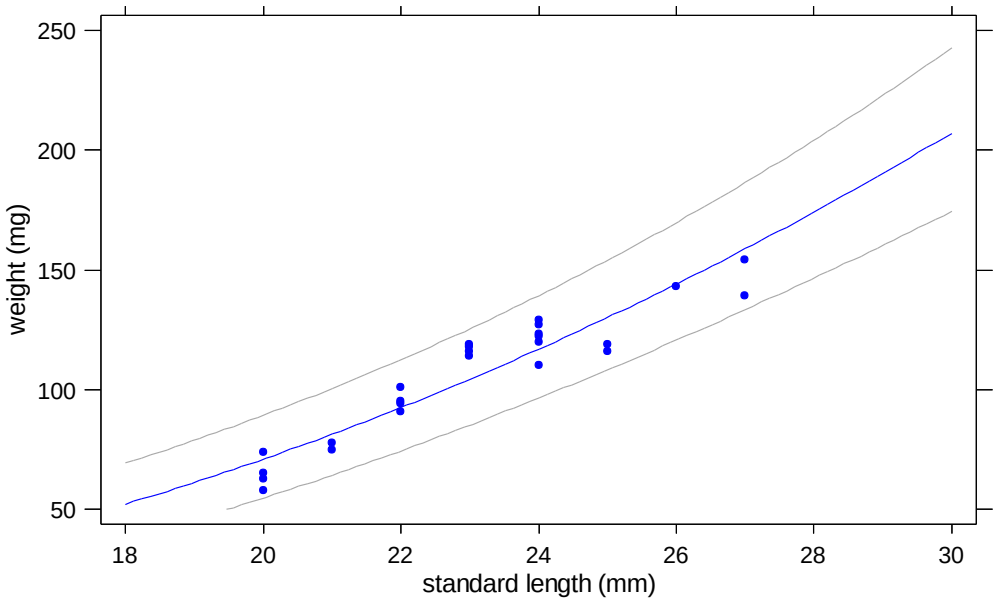


Figure 6.2: Square-root Y regression of standard length vs. weight for Alafia River *Anchoa mitchilli*, July 2011. Grey lines indicate limits of prediction for the equation. The regression line is defined by the relationship weight = $(-3.48 + 0.59 * \text{length})^2$. Adjusted $R^2 = 87.5\%$.

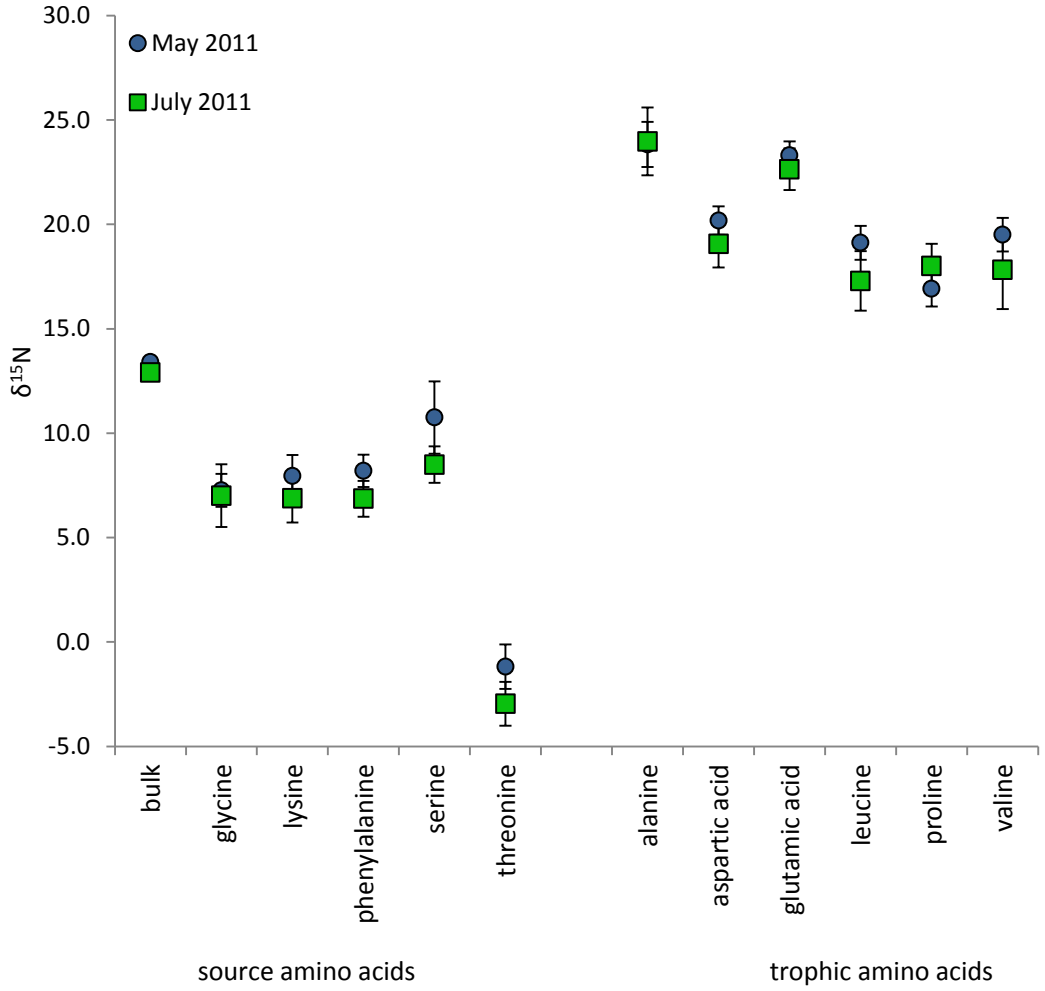


Figure 6.3: Mean amino acid $\delta^{15}N$ values for 2011 Alafia River *A. mitchilli* samples (N = 12 per season). Error bars are standard deviations between individuals within each sample. Bulk values are presented for comparison.

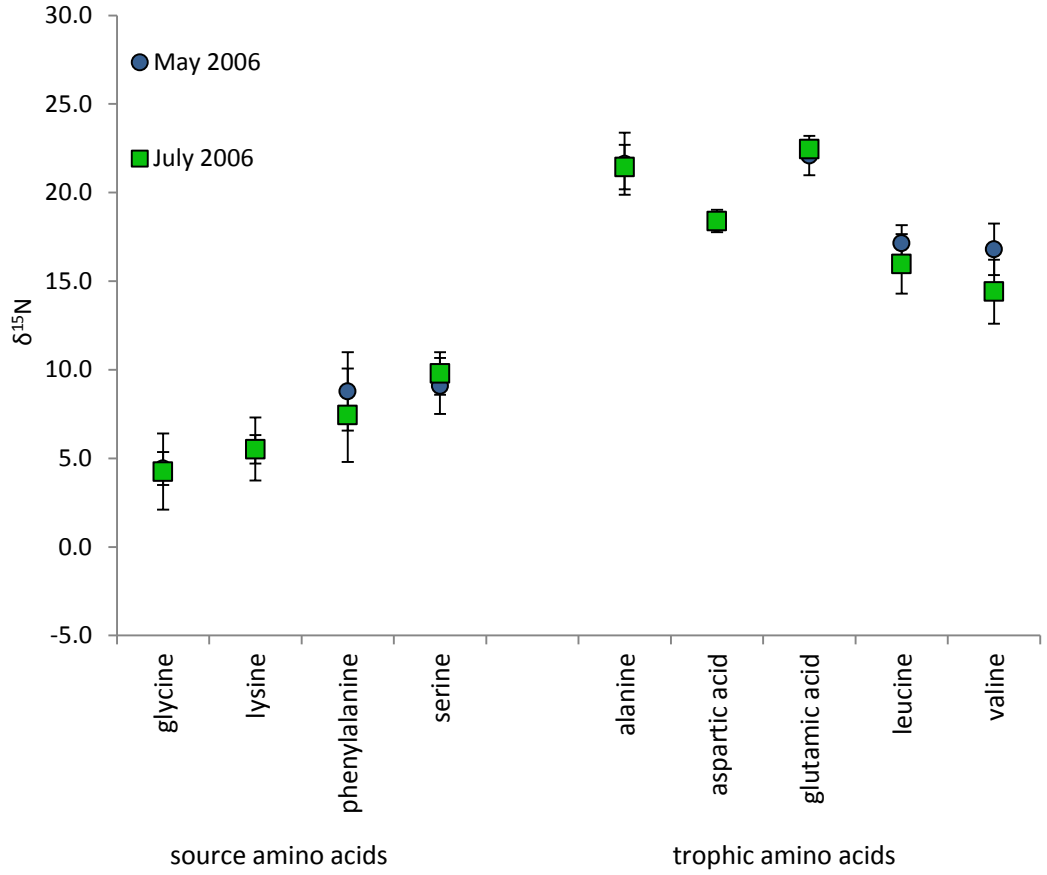


Figure 6.4: Mean amino acid $\delta^{15}N$ values for archived 2006 Alafia River *A. mitchilli* samples (N = 6 per season). Error bars are standard deviations between individuals within each sample. Proline and threonine were unresolvable due to coelution and are omitted.

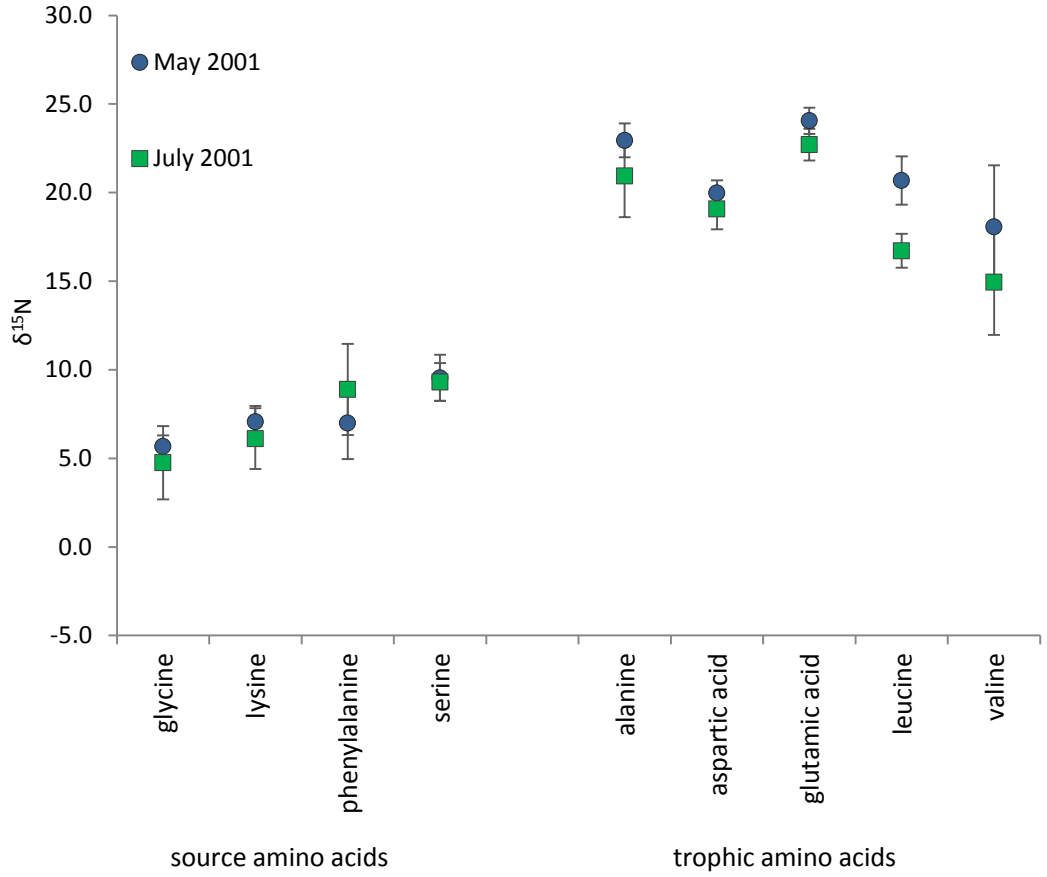


Figure 6.5: Mean amino acid $\delta^{15}N$ values for archived 2001 Alafia River *A. mitchilli* samples (N = 6 per season). Error bars are standard deviations between individuals within each sample. Proline and threonine were unresolvable due to coelution and are omitted.

Table 6.1: Length-weight data and bulk $\delta^{15}N$ values for May 2011 (dry season) and July 2011 (wet season) *Anchoa mitchilli* samples.

May 2011			July 2011		
length (std, mm)	mass (mg)	$\delta^{15}N$ (bulk)	length (std, mm)	mass (mg)	$\delta^{15}N$ (bulk)
20	103	13.19	20	65	12.77
20	80	13.07	20	63	13.07
20	88	13.33	21	75	13.21
21	97	12.44	21	78	13.34
22	115	13.25	22	91	13.28
23	113	13.22	22	94	12.48
23	119	13.43	23	114	13.48
23	137	13.51	24	127	12.14
24	148	13.10	25	116	13.21
24	129	13.30	26	143	13.11
24	143	13.29	27	154	12.94
25	161	13.33	27	139	13.01
19	70	-	20	58	-
20	79	-	20	74	-
20	89	-	22	95	-
21	92	-	22	101	-
22	109	-	23	119	-
22	114	-	23	118	-
23	118	-	23	116	-
24	126	-	24	129	-
24	147	-	24	122	-
24	160	-	24	110	-
25	161	-	24	123	-
27	204	-	24	120	-
28	213	-	25	119	-
means:					
22.7	124.6	13.21	23.0	106.52	13.00

Table 6.2: May 2011 amino acid $\delta^{15}N$ values for individual *A. mitchilli*, listed by increasing standard length. Asterisks denote "trophic II" amino acids that are thought to produce the most consistent trophic fractionations (Hannides et al. 2009). Bulk muscle tissue $\delta^{15}N$ values are listed for comparison. Amino acid-derived estimates of trophic level based on (glutamic acid - phenylalanine) and (glutamic acid - glycine) are compared in the last two columns (Hannides et al. 2009; Chikaraishi et al. 2009). Missing values indicate unresolved chromatographic peaks.

		source amino acids					trophic amino acids							
length (mm SL)	bulk $\delta^{15}N$	glycine	lysine	phenylalanine	serine	threonine	alanine*	aspartic acid*	glutamic acid*	leucine	proline	valine	TL (glu-phe)	TL (glu-gly)
20	13.2	6.7	8.6	7.0	11.6	-1.0	21.7	20.3	22.4	18.1	15.2	18.9	2.6	3.3
20	13.1	7.5	5.7	7.9	13.0	1.1	23.3	20.5	24.0	19.0	17.1	20.5	2.7	3.4
20	13.3			8.6		-1.1		19.6	22.2	20.3	19.0	18.8	2.3	
21	12.4	6.1		7.3	9.6	-0.2	22.3	20.0	22.1	17.6	16.1	18.1	2.5	3.3
22	13.3	7.4	7.7	8.3	12.4	-1.6	24.6	20.4	23.7	19.3	16.8	19.7	2.6	3.3
23	13.2	8.4	9.6	8.6	11.9	-0.6	24.6	19.0	23.1	19.0	16.6	21.0	2.5	3.1
23	13.4	6.7	8.0	7.8	11.8	-0.2	24.8	21.3	23.3	18.3	16.9	20.2	2.6	3.4
23	13.5	7.4	7.8	7.2	9.9	-2.9	22.9	20.1	23.8	19.5	16.8	18.9	2.7	3.3
24	13.1	6.2	8.1	9.5	10.2	-2.3	24.0	20.6	23.7	18.8	17.2	19.2	2.4	3.5
24	13.3	7.9	8.9	8.9	10.4	-2.0	25.2	20.5	23.6	19.2	17.4	19.8	2.5	3.2
24	13.3	8.6	8.2	8.4	11.4	-1.3	24.5	20.8	23.7	20.5	16.9	18.9	2.6	3.2
25	13.3	7.1	7.3	8.8	10.9	-2.2	24.3	19.0	24.0	19.9	17.0	20.2	2.5	3.4
means:														
22.4	13.2	7.3	8.0	8.2	11.2	-1.2	23.8	20.2	23.3	19.1	16.9	19.5	2.5	3.3

Table 6.3: July 2011 amino acid $\delta^{15}N$ values for individual *A. mitchilli*, listed by increasing standard length. Asterisks denote "trophic II" amino acids that are thought to produce the most consistent trophic fractionations (Hannides et al. 2009). Bulk muscle tissue $\delta^{15}N$ values are listed for comparison. Amino acid-derived estimates of trophic level based on (glutamic acid - phenylalanine) and (glutamic acid - glycine) are compared in the last two columns (Hannides et al. 2009; Chikaraishi et al. 2009). Missing values indicate unresolved chromatographic peaks.

			source amino acids						trophic amino acids							
length (mm SL)	bulk $\delta^{15}N$		glycine	lysine	phenylalanine	serine	threonine		alanine*	aspartic acid*	glutamic acid*	leucine	proline	valine		TL (glu-phe)	TL (glu-gly)
20	12.8		5.4	6.3	6.9	6.7	-2.6		24.6	17.6	20.9	16.7	16.2	16.2		2.4	3.2
20	13.1		7.4	8.3	6.6	8.7	-1.1		19.7	17.8	21.5	16.7	18.4	17.3		2.5	3.0
21	13.2		3.3	7.6	6.0	8.2	-1.4		23.1	17.2	21.5	16.7	18.8	13.9		2.6	3.6
21	13.3		8.3	5.1	7.6	7.7	-3.0		22.5	18.4	22.2	15.7	17.1	20.4		2.5	3.0
22	13.3		7.2		7.1	7.9	-3.8		24.4	19.3	23.1	15.8	17.9	18.3		2.7	3.3
22	12.5		7.5	7.6	7.0	8.7	-3.1		24.4	19.0	22.4	17.1	18.0	17.6		2.6	3.1
23	13.5		7.1		6.9	8.3	-3.1		25.4	19.5	22.9	16.1	18.4	17.5		2.7	3.3
24	12.1		8.8		7.1	8.2	-4.1		25.4	19.5	23.0	18.8	18.3	18.3		2.6	3.0
25	13.2		6.6	6.4	6.1	10.0	-4.0		23.9	19.1	22.7	17.4	16.2	16.1		2.7	3.3
26	13.1		8.1		8.2	9.6	-2.1		24.7	20.8	24.5	20.2	19.9	18.3		2.7	3.3
27	12.9		8.2		7.4	9.0	-2.7		25.5	20.8	23.6	19.4	18.7	20.4		2.7	3.2
27	13.0		6.2		7.2	8.9	-4.5		24.0	19.6	23.3	17.0	18.2	19.7		2.7	3.4
means:																	
23.2	13.0		7.0	6.9	7.0	8.5	-3.0		24.0	19.1	22.6	17.3	18.0	17.8		2.6	3.2

Table 6.4: Amino acid $\delta^{15}N$ values for preserved specimens of *A. mitchilli*, listed by sampling period. Asterisks denote "trophic II" amino acids that are thought to produce the most consistent trophic fractionations (Hannides et al. 2009). Amino acid-derived estimates of trophic level based on (glutamic acid - phenylalanine) and (glutamic acid - glycine) are compared in the last two columns (Hannides et al. 2009; Chikaraishi et al. 2009). Missing values indicate unresolved chromatographic peaks. Proline and threonine were unresolvable and are omitted.

			source amino acids					trophic amino acids						
period	length (mm SL)		glycine	lysine	phenylalanine	serine		alanine*	aspartic acid*	glutamic acid*	leucine	valine		TL (glu-phe)	TL (glu-gly)
May 2001	28			6.7	9.0	9.4		21.9	19.9	24.1	20.8	19.8		2.5	
May 2001	27			8.3	7.2	7.5			19.3	23.4	20.7	12.7		2.7	
May 2001	27		5.2	7.4	4.5	11.1		23.1	20.2	25.0	20.4	20.2		3.3	3.8
May 2001	25			7.1	9.5	9.4			19.7	24.4	21.3	16.6		2.5	
May 2001	28			5.6	6.5				19.3	23.0	18.3			2.7	
May 2001	25		6.1	7.3	5.2	10.2		23.8	21.3	24.4	22.5	21.1		3.1	3.6
July 2001	30		2.3	7.7	10.8	9.7		20.3	19.6	22.7	17.5	15.4		2.1	3.9
July 2001	33		6.1	7.5	9.0	8.7		24.5	20.8	24.2	17.9	18.9		2.6	3.6
July 2001	28														
July 2001	30			4.8	5.2	8.1		18.9	18.2	22.1	15.8	12.5		2.8	
July 2001	28		3.9	4.5	10.6	9.2		19.1	18.0	21.9	16.5	11.5		2.1	3.6
July 2001	29		6.8			10.8		21.8	18.8	22.6	15.9	16.3			3.3
May 2006	35		5.4	4.4	10.8	7.0		20.0	18.0	21.5	18.5	17.4		2.0	3.3
May 2006	33		4.0	3.3	8.2	8.2		23.3	17.8	20.5	15.7	15.5		2.2	3.4
May 2006	33		2.8	6.8	5.6	10.7		19.5	18.2	23.3	17.2	14.7		2.9	3.9
May 2006	35		4.7	6.6	7.0	10.7		23.9	18.6	23.3	18.2	18.8		2.7	3.7
May 2006	33		5.0	4.2	9.9	10.0		21.9	18.4	21.5	16.6	17.0		2.1	3.4
May 2006	43		4.6	7.9	11.1	8.0		21.1	18.5	22.4	16.6	17.4		2.0	3.5
July 2006	25		6.1	4.5	9.8	8.8		21.7	18.7	21.9	16.6	12.7		2.1	3.3
July 2006	28		1.4	6.7	9.9	9.6		21.1	18.6	22.6	17.6	17.1		2.2	4.0
July 2006	28		5.2	5.6	3.5	11.4		22.8	17.9	22.7	17.1	15.5		3.1	3.5
July 2006	25		6.0	5.4	6.9	10.4		22.9	18.7	22.9	16.2	15.2		2.7	3.4
July 2006	30		2.6	5.4	9.1	10.5		20.0	19.1	21.9	15.5	12.9		2.2	3.8
July 2006	29				5.4	8.1		20.1	17.4	22.7	12.9	13.0		2.8	
means:															
May 2001	26.7		5.7	7.1	7.0	9.5		22.9	20.0	24.1	20.7	18.1		2.8	3.6
July 2001	29.7		4.8	6.1	8.9	9.3		20.9	19.1	22.7	16.7	14.9		2.4	3.6
May 2006	35.3		4.4	5.5	8.8	9.1		21.6	18.3	22.1	17.1	16.8		2.3	3.5
July 2006	27.5		4.3	5.5	7.4	9.8		21.4	18.4	22.4	16.0	14.4		2.5	3.6

Chapter 7: Summary conclusions

Amino acid isotopic analysis is an emerging tool for ecological, archaeological, and paleontological studies. Nitrogen isotopes present the most promising avenue to pursue, as the predictable differences in isotopic discrimination observed between source and trophic amino acids provides an objective measure of both an organism's trophic position and the ecosystem baseline nitrogen isotope values supporting it. This information cannot otherwise be obtained from a single sample, and addresses a long-standing handicap of conventional $\delta^{15}N$ –based trophic assessments (Post, 2002; Casey and Post, 2011). Widespread application of this technique has not been achieved due in large part to methodological difficulties involved with it. This study has sought to test the applicability of amino acid-specific isotopic measurements to a series of individual problems and in so doing, to both increase understanding of the technique and to demonstrate a specific analytical methodology that is robust and repeatable.

7.1: Instrumentation

The modifications to the gas-chromatographic-combustion-mass spectrometer interface undertaken as part of this work have shown themselves to function as reliably as the original commercial kit, with less maintenance needs and at lower cost. Combined single-reactor quantitative combustion and reduction were regularly achieved, simplifying the analyate flow path and enhancing overall chromatographic

performance. Given the narrow threshold of acceptable instrument performance in GC-C-IRMS in general and in nitrogen isotope work in particular, this result is probably as significant as any of the actual experimental results.

7.2: Amino acid isotopic behavior overview

A total of 11 amino acids were reliably analyzed in nearly all applications during this study. The others were either lost during protein digestion, unsuited to the derivatization procedures, or not present in concentrations sufficient for detection. As expected, trophic amino acids (major participants in cellular nitrogen transactions and their derivatives, such as aspartic and glutamic acid, alanine, valine, leucine and proline) showed $\delta^{15}N$ enrichment with increasing trophic level. They were also uniformly more δ^{15} N-enriched in all samples that were source amino acids (compounds undergoing little or no transamination, including glycine, lysine, phenylalanine, and serine). The latter compounds were faithful indicators of ecosystem nitrogen isotope values, with minimal changes observed across trophic levels or between species.

Threonine showed a sharp negative nitrogen isotopic fractionation with increasing trophic level across all experiments. Highly depleted values have been shown for this compound previously in literature, although it is not commonly reported due to chromatographic difficulties when using several of the more common derivatization procedures (Hare et al., 1991; Hannides et al., 2009). The mechanisms underlying this behavior are entirely unclear and are worthy of further investigation. The current paradigm for understanding of amino acid N fractionation involves only enrichment

through transamination and excretion of ^{15}N-depleted waste products. Why ^{15}N is preferentially lost from this compound cannot be explained in this framework. The lack of a transaminase enzyme for this compound (it is one of only two amino acids known to never participate in transmaminations) is likely involved, but exactly how is unclear.

7.3: Compound-specific shell organic matter analysis

Both carbon and nitrogen isotopic analysis of biomineral-bound organic matter archives were shown to benefit from the use of compound-specific analysis. The former demonstrated that environmentally relevant variability in carbon supply was faithfully recorded in individual amino acid isotopic compositions. This enhanced to scope available to resolved inter-sample differences relative to bulk measurements while also providing a pathway to potentially assessing the effects of diagenesis on ancient materials.

Amino acid δ^{15}N analysis of shell organic matter provided a means to overcome ambiguity in bulk trophic determinations caused by species-specific isotopic offsets. These offsets precluded reconstructing inter-species trends in isotope values seen in soft tissue samples, but were not a factor in compound-specific measurements. Ecosystem baselines and individual organism trophic positions could both be accurately estimated across the entire suite of species studied.

7.4: Trophic position and ontogenetic diet shifts

Understanding of the timing of ontogenetic diet shifts in juvenile fishes is an important tool in managing commercially valuable species, as these changes are determinants of recruitment success and drivers of eventual adult stock biomass. The ability to determine baseline-independent trophic estimates on individual samples allows detection of ontogeny-driven dietary patterns in fish populations without the need for labor-intensive gut content analyses. The population of *Bairdiella chrysoura* analyzed in this study showed that CSIA-derived trophic measurements accurately capture evidence of known dietary changes with increasing body size. The extent of trophic level increases indicated by the isotopic results between juvenile and adult year classes was also consistent with pre-existing gut content-derived estimates. The precision and relative ease of this technique should provide a useful tool for fisheries researchers and managers.

7.5: Sensitivity of amino acid $\delta^{15}N$ to diet quality

One of the most promising aspects of amino acid-based estimates of trophic position is the ability to extract useful environmental information from a single sample with no a priori knowledge of its environmental history. The isotopic distance between source and trophic amino acids in $\delta^{15}N$ space has been put forward as a means of estimating absolute trophic positions without any independent measure of ecosystem nitrogen isotope baseline values. In order for this to be possible, it is essential that the extent of compound-specific fractionation be precisely known and stable across species and

environmental conditions. This latter requirement, while sometimes assumed, is by no means assured.

Comparison of samples of *Anchoa mitchilli* drawn from the same environment but with significantly different nutritional conditions showed that disparities in health (as measured by length-weight relationships) had no detectable impact on mean source-trophic amino acid offsets in $\delta^{15}N$. This suggests that these patterns are in fact reasonably stable across a fairly wide range of environmental conditions and as such can produce reliable trophic estimates in many different circumstances.

7.6: Areas of future research

The results obtained here suggest three main areas where further understanding is needed for wider application of amino acid isotopic studies. Firstly, improvements in chromatographic performance to eliminate co-elution and increase separation between analyate peaks would benefit these types of measurements, which are made near the limits of performance of the underlying instruments. Closely related to this is the need to perform rigorous comparisons of different derivatization methods to insure that all produce equivalent, or at least directly comparable, results.

Second, further inquiry is urgently needed into the effects of environmental variability on compound-specific fractionation factors. It is an often-overlooked fact that bulk trophic isotopic fractionation factors are in reality quite variable (Minagawa and Wada, 1984). This variation must come from changes on the level of individual amino acids, yet existing formulas for trophic estimation based on amino acid $\delta^{15}N$ entirely

discount it (Chikaraishi et al., 2009; Hannides et al., 2009). The results obtained here with *Anchoa* suggest that within certain thresholds this simplification appears to be valid, but it still remains to be tested rigorously in controlled culture experiments.

Finally, the coefficients underpinning the aforementioned formulas also require further validation. The existing set of equations produce substantially different estimates of trophic position both in this study as well as in other published datasets, yet no attempts have thus far been made to reconcile them. The most recent and widely accepted of these (Chikaraishi et al. 2009) tended to systematically under-estimate trophic levels here. Whether this was due to environmental or laboratory effects cannot be clearly determined, but should be addressed in further work.

Despite this, compound-specific amino acid analysis provides a range of capabilities not available through bulk methods and it is hoped that this utility will lead to wider acceptance and usage of this powerful technique in future research.

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